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METABOLISM OF D-GLUCOSAMINE*

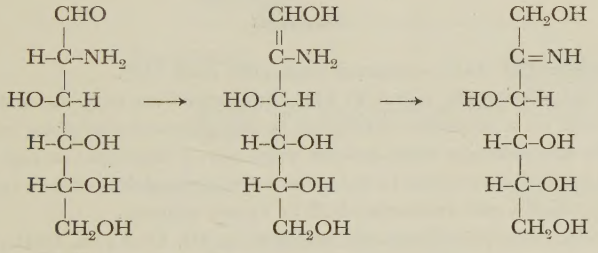
THE FORMATION OF D-FRUCTOSE-6-PHOSPHATE

By YUJIRO IMANAGA

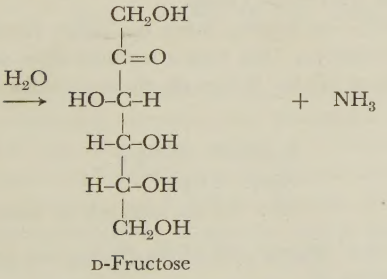
(*From the Department of Science, Nara Women's University, Nara*)

(Received for publication, September 14, 1956)

Fermentation of D-glucosamine by *Aerobacter cloacae*, producing mainly 2,3-butylene glycol, was found several years ago (*1*). As the formation of butylene glycol from natural sugar is a typical fermentation by this micro-organism (*2*), it was suggested (*1*) that glucosamine was deaminated and converted to zymohexose, probably to D-fructose, following a sort of Lobry du Bruyn (*3*) reaction.



D-Glucosamine



D-Fructose

The study on glucosamine metabolism, having achieved a remarkable development in these several years, found phosphate of sugar to

* Reported at Symposium on Enzyme Chemistry (Tokyo Univ.), July (1956)

play certain roles (*4*~*14*), and made it probable that the above suggested reaction might proceed via phosphoric esters.

The author has proved the conversion of glucosamine-6-phosphate to fructose-6-phosphate by an enzyme preparation from *Aerobacter cloacae*. Decomposition of free glucosamine by the enzyme preparation is very slow, but it is greatly accelerated by the addition of adenosinetriphosphate (ATP). Therefore, the enzyme preparation must contain not only phosphoglucosamine isomerase but also glucosamine phosphokinase.

A report from Buenos Aires (*15*), revealing the formation of fructose-6-phosphate from glucosamine-6-phosphate by animal tissues, was brought during preparation of this manuscript to draw the author's attention to that higher animals as well as micro-organism metabolize D-glucosamine.

EXPERIMENTAL

Materials

D-Glucosamine-HCl (GA)—prepared from crab shell (*16*).

D-Glucosamine-6-phosphate (GA-6-P) (*13*)—prepared from D-glucosamine and tetraphosphoric acid by a procedure analogous to the glucose-6-phosphate synthesis, and purified as Ba salt, yielding a white powder which gave a single spot on paper chromatograph by 0.2 per cent ninhydrine in water-saturated butanol, Elson-Morgan reagent, molybdenum reagent and ammoniacal silver nitrate solution.

Glucose-6-phosphate (G-6-P)—kindly supplied by Dr. Uehara, Osaka University.

Adenosinetriphosphate (ATP) *and Protamine*—kindly supplied by Dr. Suda, Institute of Microbial Disease, Osaka University, Osaka.

Fructose-6-phosphate (F-6-P)—on market, made by Tokyo Kasei Kogyo K. K.

Besides the above abbreviations, TCA means trichloroacetic acid.

Aerobacter cloacae—grown at 37° for 3 days on glucose-bouillon-agar, composition of which was as follows:

Glucose: 10.5 g.,	Peptone: 10.5 g.,
Bouillon: 10.5 g.,	Agar: 21 g.,
NaCl: 1.05 g.,	pH: 7.0 in 1,000 ml. of water.

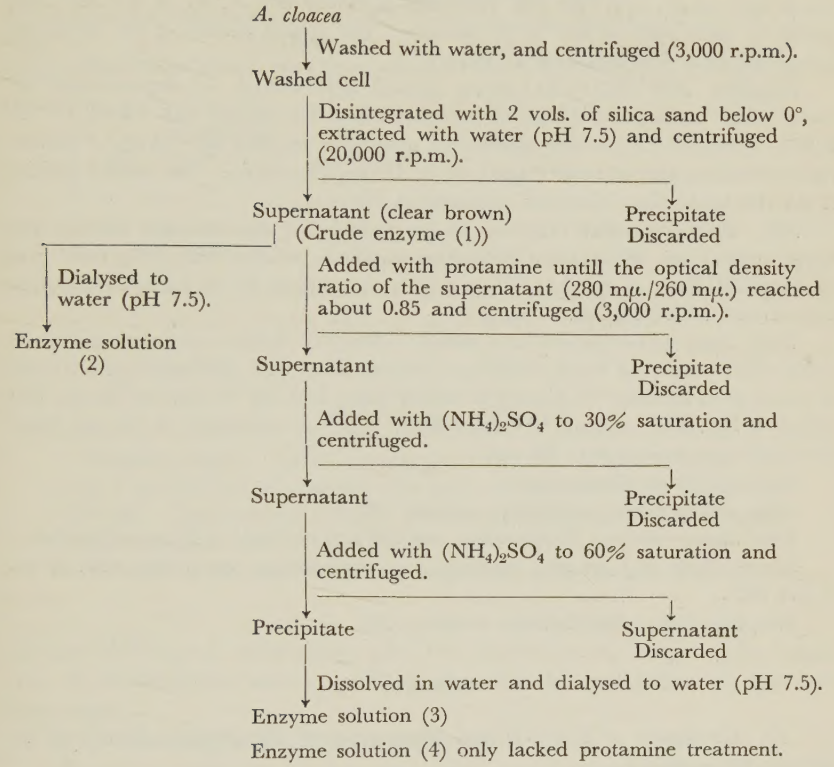
Preparation and Purification of Enzymes—all of the purification procedures shown in Table I were carried out below 5°.

Analytical Methods

Enzymatic studies were carried out by main and control experiments. The latter,

TABLE I

Preparation and Purification of Enzymes



lacking substrate, was done in parallel to the former, the substrate being added with TCA or other deproteinizing reagents at the termination of the reaction.

GA and GA-6-P (Elson-Morgan method) (17)—1.0 ml. of deproteinized solution which contained about 0–0.5 μM of GA or 0–0,8 μM of GA-6-P was mixed with 1.0 ml. of 0.2 per cent acetylacetone in 1 *N* sodium carbonate solution, and the mixture was heated in water bath at 89–92° for 45 minutes, cooled, and 2.5 ml. of alcohol, 1.0 ml. of Ehrlich's reagent* and then 4.5 ml. of alcohol were added and kept at 37° for 20 minutes. Optical density of violet-red color developed was measured at 535 mμ. The rate of decrease of the amino sugar was calculated from the optical densities of the

* 800 mg. of *p*-dimethylaminobenzaldehyde dissolved in 15 ml. of alcohol, mixed with 15 ml. of conc. HCl.

main and control experiments.

Ketose and Ketosephosphate (*Roe method*) (*18*)—1.0 ml. of deproteinized solution was mixed with 1.0 ml. of 0.1 per cent resorcinol in alcohol and 3.0 ml. of 6 *N* HCl, and heated in water bath at 80° for 20 minutes. The optical density of the developed orange-red color was measured at 485 mμ.

Phosphoric Acid (*Fiske-Subbarow method*) (*19*)—2.0 ml. of deproteinized solution which contained 0–1.0 μM of phosphoric acid was mixed with 1.0 ml. of 3 *N* H_2SO_4, 1.0 ml. of 2.5 per cent ammonium molybdate solution and 0.4 ml. of aminonaphtholsulfonic acid solution** and kept at 37° for 15 minutes. The optical density of the developed blue color was measured at 720 mμ.

NH_3 (*Conway method*) (*20*)—a proper quantity of deproteinized solution was mixed with 1.0 ml. of saturated pottasium carbonate solution and NH_3 freed was absorbed by 1.0 ml. of *N*/100 H_2SO_4 in a Conway dish at 37° for 3 hours. H_2SO_4 was titrated with *N*/100 NaOH.

Total Sugar (*Somogyi-Nelson method*) (*21*) (*22*)—2.0 ml. of deproteinized solution which contained 0–0.6 μM of sugar (evaluated as GA) was mixed with 2.0 ml. of Somogyi's solution***, heated in boiling water bath for 10 minutes, cooled, and 2.0 ml. of Nelson's reagent**** was added. The optical density of the developed olive color was measured at 500 mμ.

Color Reagent for Chromatography.

Amino Sugars—Elson-Morgan reagent (*23*).

Total Sugars—300 mg. of benzidine and 200 mg. of TCA in 25 ml. of alcohol.

Ketose—10 ml. of 1 per cent resorcinol in alcohol solution was mixed with 90 ml. of 2 *N* HCl.

Phosphoric Esters—Molybdenum reagent (*24*).

RESULTS

1) Conversion of GA-6-P and Suppression of Phosphatase Activity of the Enzyme Preparations—

Cysteine had little effect, while NaF inhibited definitely the phosphatase activity of both dialysed crude and partially purified enzyme preparations and, moreover, accelerated greatly the decrease of GA-6-P

** Solution of 500 mg. of aminonaphtholsulfonic acid in sodium bisulfite solution (15 g./100 ml.), mixed with 5.0 ml. of sodium sulfite solution (1.0 g./5.0 ml.).

*** 24 g. of sodium carbonate and 12 g. of Rochelle salt, dissolved in about 250 ml. of water, added (with stirring) by 40 ml. of 10 per cent cupper sulfate solution, followed by addition of 16 g. of sodium bicarbonate, mixed with sodium sulfate solution (18 g./500 ml.) and diluted to 1,000 ml.

**** 25 g. of ammonium molybdate, dissolved in 450 ml. of water, mixed with 21 ml. of conc. H_2SO_4, and added by solution of 3 g. of sodium arsenate in 25 ml. of water.

TABLE II

GA Degradation and Phosphatase Activity

Exp. No.	Enzyme	Inhibitor	Hydrolysis of phosphoric ester (%)	Decrease of GA–6–P (%)	Formation of NH_3 (%)	Ketose* Opt. D. (485 mμ)
1	(2)	Cysteine	49.5	16.5		
		.....	52.0	17.0		
2	(2)	NaF	11.0	67.5	49.5	Main 0.167
						Control 0.060
		.....	63.0	17.0	18.0	
3	(3)	NaF	0.5	81.0		
		.....	37.5	64.4		

Complete system: 2.0 ml. of enzyme, 1.4 ml. of *M*/5 borate buffer (pH 7.5), 8 μM (0.4 ml.) of substrate (GA-6-P), 0.2 ml. of inhibitor (final conc. 10^{-2} *M*). Total volume, 4.0 ml. The incubation was at 37°, for 3 hours. Deproteinization with TCA.

* 1.0 ml. of the total 4.9 ml. was submitted to estimation (Fig. 2).

(Elson-Morgan coloration) and the formation of NH_3. The ketose test of the reaction mixture clearly showed the formation of fructose-like sugar.

2) Chromatography of Reaction Mixtures—

Reaction mixtures of the main and control experiments (Table II, Exp. 3) were concentrated in vacuo and subjected to paperchromatography with tert. butanol-formic acid-water solvent system (3 : 1 : 1). The results are shown in Table III.

A new spot which moved faster than GA-6-P was observed clearly. By its positive reaction to benzidine-TCA, resorcinol-HCl and molybdenum reagent, and by its negativity to Elson-Morgan's reaction, it is apparent that GA-6-P was converted to a ketose-phosphate.

3) Isolation and Identification of F-6-P—

The isolation procedure are shown in Table IV.

Paperchromatograms of the isolated product are shown in Fig. 2.

4) Optimum pH of Phosphoglucosamine Isomerase—

The optimum pH was about 7.5 (Fig. 3).

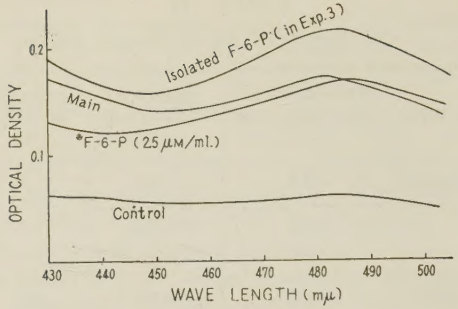


FIG. 1. Absorption spectra of Setiwanow reaction of ketose.
* F-6-P was synthesized from fructose-1,6-di-phosphate (*25*).

TABLE III

Chromatography of Reaction Mixture

Developing reagents	Substances to be detected	R_f Values			Colors developed
		Main	Control	Standard GA-6-P	
Elson-Morgan	Amino sugars	0.08 (Very faint)	0.09	0.09	Pink
Benzidine-TCA	Sugars in common	0 14	0.10	0.08	Brown
Resorcinol-HCl	Ketoses	0.19	Negative	Negative	Red
Molybdenum-reagent	Phosphoric esters	0.12			Blue
		(Rg* (R_f (Unknown)/ R_f (Glucose))			
Benzidine-TCA	Sugars in common	0.72	0.35		Brown
Resorcinol-HCl	Ketoses	Positive	Negative		Red

* The solvent was overflowed beyond the end of the paper strips for about 20 hours, using glucose as the standard.

5) Specificity of Isomerase and Phosphatase—

TABLE IV

Isolation of F-6-P

The reaction mixture contained 14 ml. of enzyme (3) opt. D. ratio (280 mμ./260 mμ.): 1.20), 144 μM (5.4 ml.) of GA-6-P, 1.6 ml. of NaF solution (final conc. 10^{-2} *M*) and 11 ml. of *M*/5 borate buffer (pH 7.5). Total vol. was 32 ml. The incubation was at 37° for 4 hours. After deproteinization with 4.0 ml. of 10 per cent TCA solution, the clear supernatant (GA-6-P Decrease: above 90 per cent) was treated as follows.

Supernatant

↓ Mixed with 0.1 ml. of 48% HBr, neutralized with $BaCO_3$, then adjusted to pH 6.0 with 0.1 *N* $Ba(OH)_2$, and centrifuged.

Supernatant 37 ml. → Precipitate Discarded

↓ Mixed with 4 vols. of alcohol, stored overnight in ice box and centrifuged.

Precipitate → Supernatant Discarded

↓ Dissolved in 13 ml. of water and centrifuged.

Supernatant* 15.3 ml. → Precipitate Discarded

↓ Mixed with 4 vols. of alcohol and centrifuged.

Precipitate → Supernatant Discarded

White powder (40 mg.)
(F–6–P Ba salt)

* 0.5 ml. of this solution was submitted to ketose estimation (Fig. 1).

Since the ketose was not produced from G-6-P as shown in Table V, the enzyme preparation had no phosphoglucose isomerase activity. It must be concluded, therefore, that GA-6-P was converted directly to F-6-P without forming G-6-P as the intermediate.

The phosphatase activity and its inhibition by NaF were equally observed on both substrates, G-6-P and GA-6-P.

6) Kinase Activity—

The cell-free extract has almost no activity on free GA, but has high activity on GA-6-P. Remarkable decrease of free GA and free sugars by adding ATP to the system shows the phosphoryration of free GA by a kinase system (Table VI).

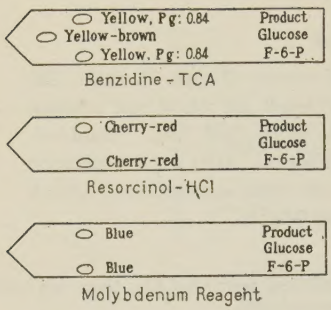


FIG. 2. Chromatogram of isolated product.

The product, glucose and F-6-P, were all dissolved in dil. HCl. Solvent system: tert. butanol-formic acid-water (3:1:1). The solvent was overflowed beyond the end of the paper strips for about 20 hours.

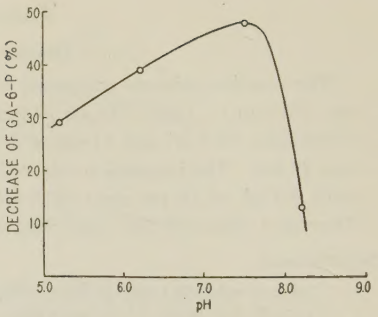


FIG. 3. pH optimum of isomerase.

Complete system: 1.5 ml. of the enzyme (IV), 1.0 ml. of $M/15$ phosphate buffer, 0.2 ml. of NaF solution (final conc., 3×10^{-2} M), 2.45 μM (0.4 ml.) of GA-6-P. Total vol., 3.1 ml. The incubation was at 37°, for 3 hours. Deproteinization with TCA.

TABLE V

Sugar Phosphate Isomerase and Phosphatase Activity of Enzyme Preparation

Substrate		Ketose* Opt. D. (485 mμ)	Decrease of GA-6-P (%)	Hydrolysis of phosphoric ester (%)	
				With NaF	Without NaF**
G-6-P	Main	0.164	——	0.5	34.0
	Control	0.178			
GA-6-P	Main	0.242	75.5	2.5	30.0
	Control	0.097			

Complete system: 2.5 ml. of enzyme (4) opt. D. ratio (280 mμ./260 mμ.: 0.85), 0.9 ml. of $M/5$ borate buffer (pH 7.5), 0.2 ml. of NaF solution (final conc. 10^{-2} M), 16 μM (0.4 ml.) of substrate. Total vol., 4.0 ml. The incubation was at 37°, for 3 hours.

* After stopping the reaction with TCA, 1.0 ml. of the total 5.0 ml. was submitted to estimation.

** The system contained 1.5 ml. of enzyme (4), 0.6 ml. of $M/5$ borate buffer (pH 7.5), and 1.6 μM (0.4 ml.) of substrate. Total vol., 2.5 ml.

TABLE VI

GA Phosphokinase Activity

Enzyme	Buffer solution	Addition of ATP	Decrease of GA (%)	Decrease of free sugars (%)	Substrate (GA) used (μM)
(1)	*M*/15 Phosphate	+	76.0		2.7
		−	10.0		
(1)*	*M*/5 Borate	+	29.5	25.5	4.0
		−	7.0		
(2)	,,	+	12.5		4.0
		−	− 6.0		
(4)**	,,	+	7.5		2.7
		−	− 2.0		

Complete system: 1.5 ml. of enzyme, 0.9 ml. of buffer solution (pH 7.5), 0.6 ml. of NaF solution (final conc. 3×10^{-2} *M*), 0.2 ml. of $MgSO_4$ solution (final conc. 5×10^{-3} *M*), 7 μM (0.4 ml.) of ATP, 0.2 ml. of GA solution. Total vol., 3.8 ml. The incubation was at 37°, for 3 hours. Deproteinization with TCA.

* Complete system: 2.0 ml. of enzyme, 1.0 ml. of buffer solution (pH 7.5), 0.2 ml. of NaF solution (final conc. 3×10^{-2} *M*), 0.2 ml. of $MgSO_4$ solution (final conc., 5×10^{-3} *M*), 7 μM (0.4 ml.) of ATP, 0.2 ml. of GA solution. Total vol., 4.0 ml. The incubation was at 37°, for 3 hours. Deproteinization with 1.0 ml. of 5 per cent $ZnSO_4$ and 1.0 ml. of 0.3 *N* $Ba(OH)_2$ solution.

** Decrease of GA-6-P by this enzyme preparation was 49.5 per cent. (The system: 1.5 ml. of enzyme, 1.0 ml. of *M*/5 borate buffer (pH 7.5), 0.6 ml. of NaF solution (final conc., 3×10^{-2} *M*), 0.6 ml. of GA-6-P (4.0 μM). The incubation was at 37°, for 3 hours.

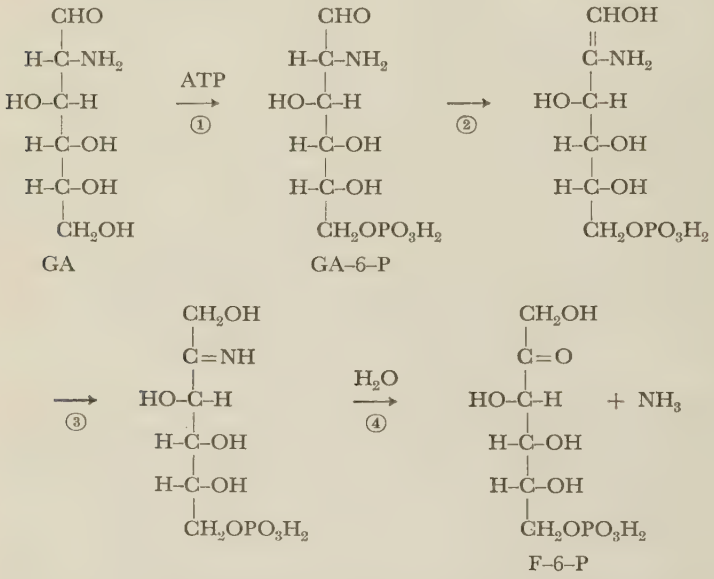
DISCUSSION

GA is easily decomposed by alkali forming NH_3. As it is well known that aldose is converted to corresponding ketose in basic medium (Lobry du Bruyn reaction), the decomposition of GA by alkali is supposed to proceed in the analogous way in which isomerization is followed by hydrolysis forming NH_3 and the ketose thus formed is degraded further.

The results obtained by K. Heyns (*26*) that glucosamine was

formed from fructose and NH_3 and glucose could hardly replace fructose show the probability of the above conversion to proceed in the reverse direction.

In this article, conversion of GA-6-P to F-6-P with NH_3-formation by a cell-free extract of *Aerobacter cloacae* has been clarified. Addition of ATP enabled crude extract to phosphorylate free GA for decomposition of it. Hence, also in the intact cell, GA may be supposed to decompose in the analogous way to glucose break down through phosphokinase and phosphoglucose isomerase reaction.



Reaction 1) of the above process has been shown by several workers ((*7*)~(*11*), (*13*)).

Reactions 2), 3) and 4) are hypothetical, however, the findings that our enzyme preparation exhibited no phosphoglucose isomerase activity and, acting anaerobically also, could be dialyzed without loss of activity, make it unprobable that GA-6-P is deaminated by direct hydrolysis or oxidoreduction.

For the purpose of the most reasonable explanation of the reactions the author suggests the presence in this enzyme preparation of a new enzyme " Phosphoglucosamine isomerase " which catalyzes reaction 2)

and 3) of the above process. Reaction 4) is presumably spontaneous.

The deamination process in this schema is one which follows the isomerization. Such a type of deamination may be one of new character in comparison with the usual oxidative (*cf.* amino acid oxidase) or intramolecular deamination (*cf.* aspartase, histidine deaminase).

SUMMARY

1. A cell-free extract from *Aerobacter cloacae* has been found to deaminate GA-6-P and the enzyme is partially purified. In the following reaction,

$$\text{GA-6-P} \longrightarrow \text{F-6-P} + \text{NH}_3$$

F-6-P has been isolated as Ba salt and identified chromatographically.

2. Crude extract is capable of phosphorylating free GA by the addition of ATP to the system. Kinase activity decreases by dialysis or by ammonium sulfate fractionation.

3. The enzyme preparation shows phosphatase activity towards GA-6-P and G-6-P and is inhibited by NaF (10^{-2} *M*). Cysteine has no inhibiting effect upon the hydrolysis of GA-6-P.

4. A mechanism of GA catabolism and a new type of enzymic deamination are proposed.

The author takes liberty of expressing his thanks to Prof. S. Akabori of Osaka University and Prof. Y. Matsushima for their kind guidance, and also to Misses Y. Kusanagi, S. Yokoyama, Y. Yamada, T. Oya, K. Maeda, M. Abu, Y. Sekiya, S. Ogura and M. Mikami for their assistance.

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MICROBIOLOGICAL DEGRADATION OF BILE ACIDS
IX. FORMATION OF 7α,12α-DIHYDROXY-3-KETO-Δ^4-CHOLENIC ACID FROM CHOLIC ACID BY A SOIL BACTERIUM

By TAKEO EGUCHI

(*From the Departments of Biochemistry and Bacteriology, Okayama University Medical School, Okayama*)

(Received for publication, September 26, 1956)

In the previous papers (*1–2*) of this series, it has been demonstrated that *Streptomyces gelaticus* 1164 was able to grow on a medium containing cholic acid as the sole source of carbon and convert the cholic acid into 7α-hydroxy-3,12-diketo-Δ^4-cholenic acid (*1*) and 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid (*2*).

Recently another bacterium which was also able to grow on the same medium was isolated from the soil of a slaughter-yard, on where gall bladders were usually hung for drying. The present report was designed to yield information as follows: a) on the isolation of the cholic acid-oxidizing organism from soil and b) on the degradation product of cholic acid by this organism.

Full details of the identification and further characterization of this organism will be reported before long.

EXPERIMENTAL

Isolation and Characteristics of Cholic Acid-Oxidizing Organism—A synthetic culture medium which was described in the first paper (*3*) of this series was prepared and adopted for this experiment as follows: Cholic acid 2.0 g., K_2CO_3 0.6 g., $(NH_4)_2SO_4$ 2.0 g., K_2HPO_4 1.0 g., $MgSO_4 \cdot 7H_2O$ 0.5 g. and $FeCl_3 \cdot 6H_2O$ 0.01 g. were dissolved in 1,000 ml. of distilled water and the resulting solution was adjusted to pH 7.4. The culture medium was tubed in 5 ml. quantities in small test tubes (1.0 cm. in diameter) and autoclaved at 110° for 20 minutes.

A sample of soil from the slaughter-yard above mentioned was added to a test tube containing the culture medium, covered with a thin layer of this culture medium, and then incubated at 37°. After turbidity appeared in the supernatant, the culture was transfered to another test tube containing the same medium. Several transfers were made in the same manner. Finally, by streaking on a common agar plate, several pure strains of bacteria were isolated.

One of the stains (CE-1) was used in this study. It was a Gram-positive and

non-sporulating coccus which grew well at 37° and in the range of pH 6.5 to 7.5. As indicated in a report (*4*) of this series, Fig. 1 suggested that this strain was capable of degrading cholic acid to a compound containing 7α-hydroxy-3-keto-Δ^4-ene grouping.

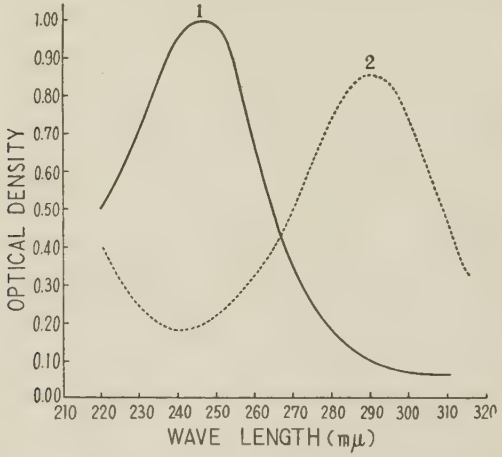


FIG. 1. Ultraviolet absorption spectra of the supernatant (centrifuged, 8,000 r.p.m. 15 minutes, and diluted 1:30) of the culture medium obtained after incubation of cholic acid (0.2 g./dl.) with strain CE-1 for 7 days at 37°. Curve 1, diluted supernatant only; curve 2, solution which was heated in a boiling water for 5 minutes with *N*-NaOH of a tenth volume of the original diluted supernatant.

Cultivation and Isolation of Degradation Products—800 ml. of the above-described synthetic culture medium in an Erlenmeyer flask was autoclaved at 110° for 20 minutes and inoculated with strain CE-1. The inoculated flask was incubated for 11 days at 37°. The pH of the culture medium dropped during the incubation to 6.4 from the original pH 7.4, but still gave a weak Pettenkofer's test. At the end of the incubation period the mixture was treated as shown in Table I.

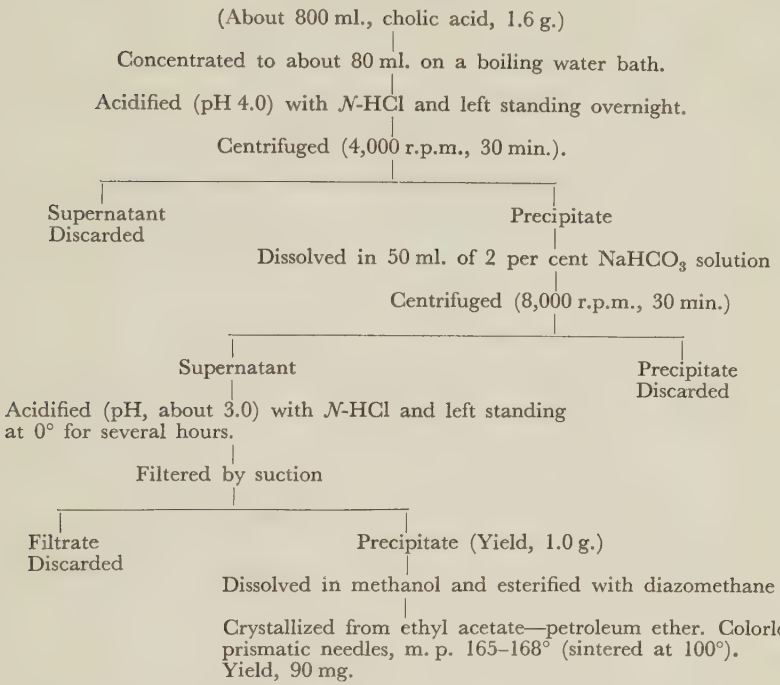
Further recrystallization of the crystals (m.p. 165–168°) thus obtained from ethyl acetate afforded plates, m.p. 178–180°, $\lambda_{max.}^{alc.}$ 242 mμ (log ε 3.97). The infrared spectrum showed the following abosorption bands (KBr disk): hydroxyl, 2.93 μ; ester, 5.75 μ; Δ^4-3-ketone, 6.03 and 6.27 μ.

Anal. Calcd. for $C_{25}H_{38}O_5$: C, 71.74; H, 9.15
Found: C, 71.56; H, 9.24.

Methyl 12α-hydroxy-3-keto-$\Delta^{4,6}$-choladienate—A solution of 20 mg. of the above ester, m.p. 178–180°, in 2 ml. of methanol containing one drop of concentrated sulfuric acid

TABLE I

Isolation of Cholic Acid Metabolites by Strain CE–1.



was refluxed on a boiling water bath for 20 minutes. The resultant yellow solution was diluted with dilute sodium bicarbonate solution and the crystalline precipitate which was separated on standing, was collected, washed with water and dried. Crystallization of the dried precipitate from methanol—water afforded 14 mg. of pale yellow prismatic needles of m.p. 182–184°, $\lambda_{max.}^{alc.}$ 284 mμ (log ε 4.42). The infrared spectrum showed the following absorption bands (KBr disk): hydroxyl, 2.857 μ; ester, 5.81 μ; $\Delta^{4,6}$-3-ketone, 6.03, 6.20 and 6.33 μ. Identity with an authentic sample (*5*) with m.p. 184–185.5° was demonstrated by mixed melting point determination and infrared comparison.

Anal. calcd. for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06.
Found: C, 74.44; H, 9.08.

DISCUSSION

A new methyl ester with m. p. 178–180°, isolated from the cholate

culture of strain CE-1, coincided with the formula $C_{25}H_{38}O_5$ containing a Δ^4-3-ketone structure from the data of the ultraviolet and infrared absorption spectra and the micro-analysis data. Also this ester afforded the known methyl 12α-hydroxy-3-keto-$\Delta^{4,6}$-choladienate by refluxing with methanolic sulfuric acid. In a previous paper (*1*), it has been demonstrated that 7α-hydroxy-3-keto-Δ^4-ene-steroids will be easily dehydrated into $\Delta^{4,6}$-3-ketone structure during esterification by methanolic sulfuric acid. In view of the above-mentioned facts the most reasonable conclusion to be drawn from available data is that the new ester with m.p. 178–180° may be methyl 7α,12α-dihydroxy-3-keto-Δ^4-cholenate.

It is also well known that the dehydration of the $C_7\alpha$-hydroxyl in 7α-hydroxy-3-keto-Δ^4-ene-steroids is easily catalyzed by both mineral acid and alkali (*2*). Therefore it is presumed that, as shown in Fig. 1, an appearance of $\lambda_{max.}^{water}$ 246 mμ in the cholate culture of strain CE-1 is due to the formation of 7α,12α-dihydroxy-3-keto-Δ^4-cholenic acid from cholic acid and the shift of $\lambda_{max.}^{water}$ from 246 to 290 mμ after alkali-treatment is attributable to a dehydration of the $C_7\alpha$-hydroxyl in 7α, 12α-dihydroxy-3-keto-Δ^4-cholenic acid to form 12α-hydroxy-3-keto-$\Delta^{4,6}$-choladienic acid. Furthermore, the above evidence indicating a bacterial oxidation of cholic acid into 7α,12α-dihydroxy-3-keto-Δ^4-cholenic acid suggests that the first oxidation product of cholic acid by strain CE-1 may be 7α,12α-dihydroxy-3-ketocholanic acid as indicated in the experiment with *S. gelaticus* 1164 (*1*), although this mono-keto-acid is not yet isolated as crystals. This is the first observation which demonstrates an unsaturation of the cholane nucleus of cholic acid by bacteria. On the metabolism of cholic acid by microorganisms, a significance of the formation of an intermediate containing 7α-hydroxy-3-kto-Δ^4-ene grouping from cholic acid has been proposed through the experiments with *Streptomyces spp.* in an earlier paper (*4*). In this paper, it is also presumed that a bacterium (strain CE-1) as well as *Streptomyces app.* has such a transformation pathway for cholic acid and the newly produced 7α,12α-dihydroxy-3-keto-Δ^4-cholenic acid will suffer a further oxidative degradation apparently including a β-oxidation of the side chain from strain CE-1.

SUMMARY

1. Some bacterial strains which can utilize cholic acid as the sole source of carbon for growth were isolated from soil.

2. Through the determination of the ultraviolet absorption spectra, it was demonstrated that one of the strains (CE-1) was capable of converting cholic acid into intermediates containing 7α-hydroxy-3-keto-Δ^4-ene grouping.

3. One of the intermediates which was isolated as its methyl ester from the culture filtrate was probably 7α,12α-dihydroxy-3-keto-Δ^4-cholenic acid.

In conclusion, the author wishes to express his sincere thanks to Dr. T. Shimizu, Dr. S. Mizuhara, Dr. S. Murakami and Dr. S. Hayakawa for their kind guidance throughout this research, and to Mr. M. Seki of Department of Chemistry, Faculty of Science, Nagoya University, for his help in measuring the infrared absorption spectra.

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STUDIES ON AMYLASE FORMATION BY BACILLUS SUBTILIS

III. NATURE OF THE ACTIVE FACTOR NECESSARY FOR THE FORMATION OF AMYLASE IN THE LYSOZYME LYSED-CELL PREPARATION

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In a previous paper it was reported that the whole lysate obtained by the treatment of *Bacillus subtilis* cells with lysozyme can still form amylase if boiled cell extract of cells cultivated in the phase of active amylase production is added (*1*). The factor discovered in this boiled cell extract has also an activating effect on the formation of amylase by intact cells (*1*), (*2*).

In the current research on the mechanism of enzyme formation, there are two major problems to be elucidated, that is, (a) the nature of the precursor material which is transformed into active enzyme molecule, and (b) the active agent (supposed to be nucleic acid) which determine the specificity of enzyme molecule synthesized. In view of the fact that the active factor in the boiled cell extract is a non-dialisable, high molecular weight substance, the following question naturally arises: whether the active factor is a precursor of amylase protein or an activator like nucleic acid which is currently presumed to be a necessary agent for enzyme formation. Therefore isotopic experiment was undertaken to answer this question and the results obtained will be reported in this paper together with other relevant experimental results on the nature of this factor.

METHODS

B. subtilis H, which has been used throughout the previous studies, was also used in this investigation. Cells were grown in Soy medium at 30° for 20 to 24 hours with shaking, harvested, wahsed once with salt solution C and resuspended in the same solution. Using this washed cell suspension activity of the factor preparation was assayed by following the formation of amylase at 30°. Usually the total volume of the reaction mixture was made twice the volume of the washed cell suspension used. In

the experiment using lysozyme lysed-cell preparation, washed cell suspension was treated with crystalline lysozyme (50 μg./ml.) at 30° for 1 hour in a Monod's shaking apparatus. Resulting viscous solution was used as a whole lysate or fractionated further by two successive centrifugations at 12,000 r.p.m. (*ca.* 15,000 g.) for 20 minutes and the supernatant solution was used as S_1 fraction (*3*).

The composition of Soy medium is as follows: soluble starch 8 per cent, Na citrate 0.04 *M*, $(NH_4)_2HPO_4$ 0.15 *M*, KCl 0.02 *M*, $MgSO_4 \cdot 7H_2O$ 0.002 *M*. $CaCl_2$ 0.001 *M*, ethyl alcohol 1 per cent (V/V), soybean extract (50 g. of soybean were extracted with about 250 ml. of 0.1 per cent NaOH by boiling for 1 hour. This extract was used per 1 litre of medium), pH 7.2. Salt solution C: Na_2HPO_2 12 H_2O 0.225 *M*, KCl 0.03 *M*, $MgSO_4 \cdot 7$ H_2O 0.003 *M*, $CaCl_2$ 0.0015 *M*, pH 7.2.

Amylase was assayed either by the method of Hagihara (*4*) (expressed as $D_{405}^{30'}$) or by the method of Smith and Roe (*5*) (expressed as S-R unit). Polysaccharide was determined by anthrone reagent (*6*). Nucleic acid content was measured from a ultra-violet absorption assuming that the optical density at 260 mμ of nucleic acid solution containing 1 mg./ml. is 22 (*7*). Total amino acid was determined by the method of Stein and Moor (*8*) after the acid hydrolysis at 100° for 8 hours. Radioactivity was determined by the conventional Geiger-Müller counter with an end window. The counting data were corrected for self absorption.

RESULTS

Fractionation of Active Factor—After the discovery of active factor which is necessary for the formation of amylase in the lysozyme lysed-cell preparation, it was attempted to purify this factor, though the purpose has not yet been achieved successfully. A fractionation diagram of this factor is presented in Fig. 1. It is a non-dialysable substance(s), can be precipitated by three volumes of ethanol and also by trichloroacetic (TCA) or perchloric acid (final concentration 0.3 *N*). Therefore, it seems to be a high molecular weight substance(s). Some experimental results showing the activity of such preparations are presented in Table I and Fig. 2.

Though it is preliminary, chemical analysis was performed on various preparations. The data vary from preparation to preparation and no definite conclusion has been obtained. Some examples were listed in Table II. However, its solution shows characteristic absorption maximum at near 260 mμ. and always presents strong positive orcinol reaction (*9*). Moreover, the precipitation by TCA could effectively eliminate the hexose polysaccharide which was contained in large amounts in the original extract. Deproteinization of preparation was also performed on the preparation at the stage B_{sp}. The factor solution was

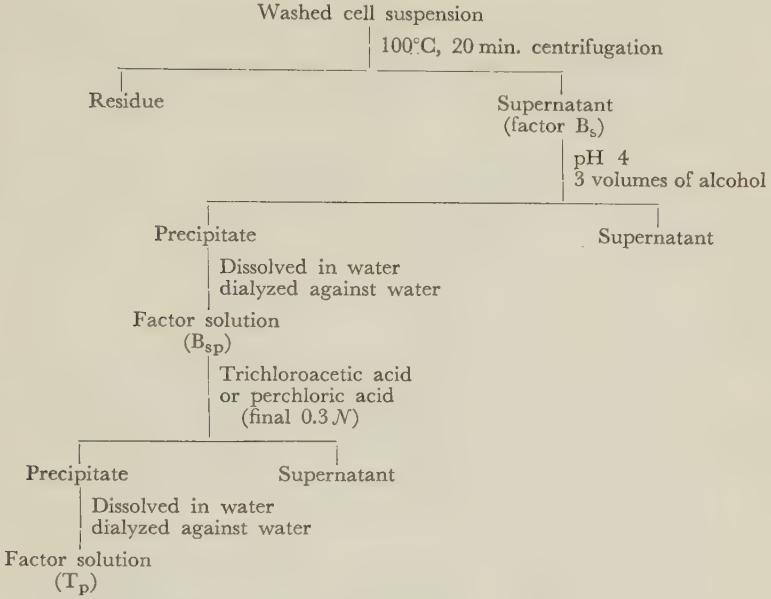

FIG. 1. Fractionation diagram of the factor.

shaken repeatedly with chloroform-butyl alcohol mixture (V/V=9:1) until no gel was formed on emulsification. To the aqueous solution finally obtained, three volumes of alcohol were added and precipitate was collected, dissolved in water and dialyzed. The preparation thus obtained still exhibited stimulating effect on the amylase formation.

Effect of Various Treatments on the Activity of Factor—Using washed cell suspension the effect of various treatment on the stimulating action of the factor was examined. Results presented in fig. 2 show that stimulating effect of factor cannot be destroyed by either ribonuclease (RNA-ase) or protease but largely destroyed by acid hydrolysis (6 N HCl, 7.5 hours).

Examination on the Precursor Nature of the Factor—Following isotopic experiments were performed in order to decide whether the factor is a precursor of amylase protein or an activator for the process of amylase formation from the endogenous material. In this case, the amylase formation by washed cell suspension was used as an experimental system.

S^{35}-Labeled cells were prepared by growing cells in S^{35}-labeled Soy medium (Table III (A)). S^{35}-Labeled factor (stage B_{sp} in Fig. 1) was

Table I

Formation of Amylase by Washed Cell Suspension, Whole Lysate and S_1 Fraction

Experimental No.		1			2						3		
Preparation		Washed cell suspension			Washed cell suspension			Whole lysate			S_1 fraction		
Glucose+amino acid mixture*		−	+	−	−	+	−	−	+	−	−	−	−
Factor B_s**		−	−	−	−	−	−	−	−	−	−	+	−
Factor B_{sp}***		−	−	+	−	−	−	−	−	−	−	−	−
Factor T_p****		−	−	−	−	−	+	−	−	+	−	−	+
Amylase ($D_{405}^{30'}$)	initial	2.5	2.5	2.5	6.4	6.4	6.4	7.5	7.5	7.5	2.7	2.7	2.7
	final	7.2	12.0	28.8	9.6	8.5	10.9	7.5	7.5	11.2	3.5	5.0	4.2
	increased	4.7	9.5	26.3	3.2	2.1	4.5	0	0	3.7	0.8	2.3	1.5

The reaction was performed at 30° in Monod's shaking apparatus for 5 hours in exp.t 1, 2 hours in expt. 2 and 6 hours in expt. 3.

* In expt. 1: glucose (1 per cent) + casein trypsin hydrolysate (4 mg./ml.). In expt. 2: glucose (0.5 per cent) + casein acid hydrolysate (4 mg./ml.) fortified with DL-tryptophan, DL-methionine (each 0.4 mg./ml.) and L-cystein hydrochloride 0.25 mg./ml.

** Its nucleic acid content is 0.55 mg./ml. in the reaction mixture.

*** Its nucleic acid content is 0.38 mg./ml. in the reaction mixture.

**** Factor T_p used in expt. 2 is that presented in Table II (sample No. 3). Final concentration in the reaction mixture is indicated in that Table. Factor T_p used in expt. 3 contains 75 μg. nucleic acid per ml. of the reaction mixture.

Table II

*Chemical Analysis of Factor Preparations**

Sample No.	Purification stage	Total amino acids	Nucleic acid	Polysaccharide
1	B_{sp}	0.21 *mg./ml.*	0.22 *mg./ml.*	50 *mg./ml.*
2	T_p	—	0.20 *mg./ml.*	5 μg./*ml.*
3	T_p	3.2 *mg./ml.*	0.07 *mg./ml.*	40 μg./*ml.*

* Figures in the Table indicate the final concentration in the test solution when the factor was demonstrated to be active in the test system.

prepared from the same S^{35}-Labeled cells as used in the experiment. Therefore, the specific activity of both S^{35}-Labeled cells and S^{35}-Labeled factor are the same. Non-labeled cells and factor were prepared in the same way and combined with the labeled factor or labeled cells as indi-

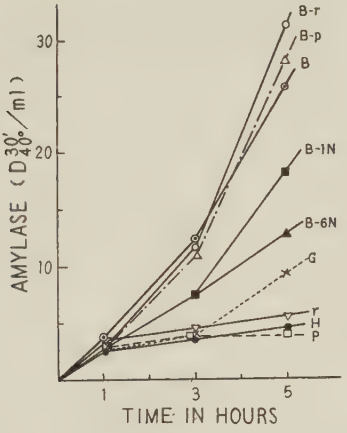

FIG. 2. Effect of various treatments on the activity of factor.

Factor preparations (B_{sp}) were treated in the following ways at 40°, for 5 hours in the presence of phosphate (*M*/100, pH 7.2). B: factor alone, B-P: factor+crystalline trypsin (210 μg./ml.)+crystalline *B. subtilis* protease (58 μg./ml.). B-r: factor+crystalline pancreatic RNA-ase (58 μg./ml.). After the treatment the reaction mixtures were heated at 100° for 2 min. Controls without added factor, *i.e.*, proteases alone (P) and RNA-ase alone (r), were prepared simultaneously. Besides the enzymatic treatment, factors were hydrolysed either with 6 *N* HCl (100°, 7.5 hours or 1 *N* HCl (100°, 1 hour), freed from HCl by vacuum-drying, dissolved in water and designated as B-6 *N* and B-1 *N* respectively. These variously treated factor preparations, which was derived from the same volume of original factor B_{sp} solution, were added to 5 ml. of washed cell suspension and made to 10 ml. with water. Formation of amylase was followed at 30°. Flask (G), which contained glucose (1 per cent) and casein trypsin hydrolysate (4 mg./ml.), and flask (H) without any addition were also prepared.

cated in Table III. After the incubation, carrier amylase was added to each of the reaction media, and crystalline amylase was isolated respectively. Isotope content of each of these crystalline amylases was determined in order to decide whether increased amount of amylase

produced due to added factor is derived from the added factor or from the endogenous material within the cell. Isolation of crystalline amylase was performed essentially in the same method as that of Hagihara (*4*). In order to avoid the possible errors due to radioactive contaminants, samples of crystalline amylase were dissolved in Ca acetate solution (*M*/100) and subjected to paper electrophoresis. The radioactivity determination and the detection of protein by Amidoschwarz 10 B were performed directly on a guide strip cut out from the end of paper. The

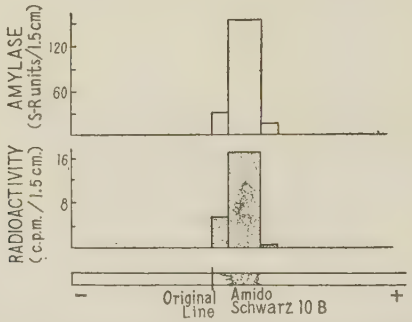

Fig. 3. Paper electrophoresis of radioactive crystalline amylase. Crystalline amylase isolated from tube C*-B (Table III) was dissolved in Ca acetate solution (*M*/100) and subjected to paper electrophoresis. Width of paper: 8 cm. Buffer: *M*/20 veronal buffer (pH 8.0) containing *M*/100 Ca acetate. Current: 0.8 mA./cm. After 4 hours run at room temperature, paper was dried in a current of air. The radioactivity determination and the detection of protein by Amidoschwarz 10 B were performed directly on a guide strip (0.5 cm. wide) cut from the end of paper. Three bands corresponding to the spot of amylase protein were cut out from the remaining paper and eluted with *M*/100 Ca acetate solution. Both amylase assay and radioactivity determination were performed on this eluate and specific activity was calculated from the value on a peak fraction.

bands of paper near those containing amylase protein were cut out and eluted by permitting Ca acetate solution (*M*/100) from a trough in a closed chamber to pass over the paper by capillary action. Both amylase assay and radioactivity determination were performed on this eluate and specific activity was calculated from the value on a peak fraction. An example of results of paper electrophoretic experiments is shown Fig. 3 and it is apparent that there is no appreciable error due to radioactive contaminants. However, the crystalline amylase derived from

C. B* had only a weak activity and could not be measured by such procedure. Therefore, its radioactivity was determined directly on

TABLE III

Isotopic Analysis of the Precursor Nature of the Factor

(A) Composition of the reaction mixture and experimental results.

	Abbreviations	C*-H	C*-B	C-H	C-B*
Composition of the reaction mixture	S^{35}-Cell suspension§	5 *ml.*	5 *ml.*	—	—
	Non-labeled cell suspension†	—	—	5 *ml.*	5 *ml.*
	S^{35}-Factor (B_{sp})§§	—	—	—	5 *ml.*
	Non-labeled factor†† (B_{sp})	—	5 *ml.*	—	—
	H_2O	5 *ml.*	—	5 *ml.*	—
Experimental results	Total amylase produced (S-R unit)	483	738	385	586
	Amylase due to added factor	—	255	—	201
	% Activation due to added factor	—	52.8	—	51.7
	Sp. Act. of amylase (c.p.m./unit)	12.6±2.1	15.5±3.6		0.34±0.06

§ Washed cell suspension prepared by growing cells in S^{35}-Soy medium at 30°, for 24 hours. Total dry cell weight 145 mg. Total radioactivity 2.54×10^6 c.p.m. The composition of S^{35}-Soy medium is same as that of ordinary Soy medium except that the amount of Na citrate and $MgSO_4\cdot7H_2O$ are reduced to 1/2 and 1/4 of ordinary amount respectively and, in addition, $MgCl_2\cdot6\,H_2O$ (0.0015 *M*) and $Na_2S^{35}O_4$ (8.3×10^{-5} *M*) are added. Specific activity of S^{35} is 7.44×10^5 c.p.m./μM S.

§§ Factor (B_{sp}) prepared from the same S^{35}-labeled cells as described above. Its nucleic acid content is 380 μg./ml. in the reaction mixture. Total radioactivity 1.89×10^5 c.p.m.

† Washed cell suspension prepared in the same way as that in the case of S^{35}-labeled cell except that $Na_2S^{35}O_4$ in the medium is replaced by $Na_2S^{32}O_4$. Total dry cell weight is same as that of S^{35}-labeled cells (145 mg.).

†† Factor (B_{sp}) prepared from the same non-labeled cells as described above. Its nucleic acid content is same as that of S^{35}-labeled factor (380 μg./ml.).

(B) Measurements of specific radioactivity of amylase isolated.

Samples of amylase	Sp. act. of amylase (c.p.m./S-R unit)
From exptl. tube C*-H	
(1) Once crystalized, then subjected to paper electrophoresis and eluted.	11.5±2.1
(2) Twice crystalized, then subjected to paper electrophoresis and eluted.	13.8±1.3
Mean	12.6±2.3
From exptl. tube C*-B	
(1) Once crystalized, then subjected to paper electrophoresis and eluted.	15.5±3.6
From exptl. tube C-B*	
(1) Once crystalized, then subjected to paper electrophoresis and eluted	—§
(2) Twice crystalized.	0.34±0.06

§ As radioactivity was very weak, measurement of sp. act. was impossible.

(C) Calculation§.

Sp. Act. of amylase in C-B*

Theory (Increased formation of amylase is assumed to be due to the conversion of a precursor contained in the factor preparation to active amylase molecule)

$12.6 \times 201/586 = 4.3$ c.p.m./S-R unit.

Observed 0.34 c.p.m./S-R unit.§§

Observed/Theory

$0.34/4.3 = 0.079$§§

Strictly speaking, as S compound contained in small amount in alkaline extract of soybean used in growth medium was not labeled with S^{35}, S^{35}-cells or S^{35}--factor might not be completely uniformly labeled. However, analysis of S^{35} of crystalline amylase obtained from this S^{35}-Soy medium and also of S^{35} of crystalline amylase obtained from uniformly S^{35}-labeled synthetic medium showed that the contribution of S in soybean extract to S of amylase protein is negligible.

§§ Specific activity of amylase obtained from tube C-B* might be less than that of observed value, 0.34, because electrophoretic purification was not performed. Therefore, true value for observed/theory might also be less than 0.079.

a crystalline sample without paper electrophoresis. The results of

these isotopic experiments are summarized in Table III (A) and (B). The calculation from these results (Table III (C)) revealed that less than 8 per cent of sulfur of amylase protein produced in response to the factor could have been derived from the factor preparation. Therefore, it was concluded that the factor is not a precursor of amylase protein but acts as an activator for the process of amylase formation from the precursor material within the cell.

DISCUSSION

From the results of isotopic experiment presented in this paper it was clearly established that the active factor in the boiled cell extract is not a precursor of amylase protein, at least with respect to its sulfur, but rather must be considered as an activator. The large amount of amylase produced in response to this active factor comes solely from the substances within the cell (or from the lysozyme lysed-cell preparation in the case of cell free system).

Hokin (*10*) reported that methionine, which was stated to be the only " essential " amino acid lacking in α-amylase of pancreas was not required for maximal synthesis of amylase by pigeon pancreas slices. However, crystalline α-amylase of *B. subtilis* H, obtained from the medium using $S^{35}O_4^{--}$ as the sole source of Sulfur, was clearly shown to contain S^{35}-methionine, but not other S-containing amino acids (*11*). This fact was also confirmed by chemical analysis (*12*). The presence of sulfur in amylase protein is, of course, clearly substantiated by the experimental results presented in this paper too (Table III and Fig. 3). Therefore, the isotopic experiment using S^{35} could be effectively performed as described in this paper. Pertinently, Caldwell *et al.* (*13*) reported that swine pancreatic amylase contains 2.1 per cent of methionine.

As to the chemical nature of the active factor, no conclusive experimental result has hitherto been obtained. The fact that the deproteinization by chloroform-butyl alcohol cannot destroy the activity, together with the fact that the activity cannot be destroyed by trypsin and bacterial protease, seems to eliminate the protein nature of the factor. Polysaccharide which was contained in large amounts in the original extract could be effectively removed by the precipitation with TCA, but, of course, it cannot make any definitive evidence against the participation of polysaccharide in the activating process. Nucleic acid or its derivative, which is currently presumed to play an important role in

protein synthesis in general, is a most likely substance to be considered. Preparations were invariably shown to contain nucleic acid or its derivatives. Moreover, the addition of yeast ribonucleic acid produced the activating effect on amylase formation in several occasions, though not always, in the system using washed cell suspension or lysozyme lysed cell preparation. However, the activity was not destroyed by RNA-ase. Therefore, the following possibility might be considered ; (a) the degradation products formed after RNA-ase action is effective. (b) Special derivative of RNA which can resist RNA-ase action, or RNA-ase resistant fraction of RNA, *i.e.*, so-called " core " is effective. (c) Substances other than nucleic acid, *i.e.*, DNA, protein, polysacchride, or contaminating small molecrelar weight coenzyme, which may be contained in the preparation are effective.

Ultimate recognition of the chemical nature of the active factor must await further experiments.

SUMMARY

The active factor in the boiled cell extract, which stimulates the formation of amylase both by washed cell suspension and in cell-free system, was partially purified.

This substance is a non-dialysable substance, can be precipitated by three volumes of ethanol and also by trichloroacetic or perchloric acid. Deproteinization by chloroform-butyl alcohol does not destroy its activity. Activity is also not destroyed by ribonuclease or protease (trypsin and *Bacillus subtilis* protease), but largely destroyed by acid hydrolysis (6 *N* HCl, 100°, 7.5 hours).

From the results of isotopic experiment using S^{35}-labeled cells and S^{35}-labeled factor preparation, it was clearly demonstrated that the active factor is not a precursor of amylase protein, at least with respect to its sulfur, but rather acts as an activator of the process.

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DEHYDROGENASES OF A HALOPHILIC BACTERIUM, WITH SPECIAL REFERENCE TO A HALOPHILIC GLUCOSE DEHYDROGENASE

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Halotolerant and halophilic bacteria were found to be a good material for the extraction of bacterial enzymes (*1*). Some of the enzymes of such bacteria presented a characteristic behavior towards the concentration of mineral salts; they exhibited the maximum activity at a very concentrated medium of mineral salts. Egami and his coworkers proposed to call them halophilic enzymes (*2*, *3*). Halophilic enzymes so far reported are alkaline phosphomonoesterase (*1*, *4*), glucose dehydrogenase (*5*), cytochrome oxidase (*6*) found in our laboratory and glycerol dehydrogenase found by Baxter and Gibbons (*7*)

As an extention of our series of biochemical studies on halotolerant and halophilic bacteria, the present research deals with the dehydrogenases of a halophilic bacterium, with special reference to the halophilic glucose dehydrogenase.

EXPERIMENTAL

Microorganism and Preparation of Enzyme Solution—A halophilic bacterium No. 101 (*Pseudomonas* species) was cultured on peptone broth agar containing 10 per cent NaCl, as previously described (*2*). The harvested cells were washed four times with 10 per cent NaCl solution and finally suspended in five volumes of distilled water.

Bacteriolysis took place in a few seconds. After stirring for 30 minutes, the suspension was centrifuged (20,000 g.) for 35 minutes at 0°. The yellowish clear supernatant thus obtained showed various dehydrogenase activities. This was used as an enzyme solution, which, when stored in 3.0 *M* NaCl solution at 3° or forzen, was almost stable at least for ten days.

Neasurement of Activity—Dehydrogenase activities were measured by Thunberg-technique with toluylene blue as a hydrogen acceptor, and expressed by the reciprocal values of decolorization time at 37°. The main tube contained 0.5 ml. phosphate buffer (1/4 *M*, pH 6.8 or 7.0), 0.5 ml. substrate (1/10 *M*), 0.5 ml. toluylene blue (1/1000 *M*) and 3.0 ml. of 5 *M* NaCl solution plus distilled water. The side arm

contained 0.5 ml. enzyme solution. The total volume of the reaction mixture was 5.0 ml.

In addition to the Thunberg-technique, a photometric determination of the enzyme, with 2,6-dichlorophenol indophenol, was employed with glucose as substrate. The components of the reaction mixture in a test tube were the same as that in Thunberg-technique except a hydrogen acceptor. After the temperature attained 37°, the reaction was intiated by adding 0.5 ml. of 2,6-dichlorophenol indophenol solution. After a definite interval, 5.0 ml. of amyl acetate was added to the reaction mixture and the unreduced dye was instantly extracted by shaking vigorously for several seconds. After about 30 minutes, amyl acetate layer was filtered. The red filtrate was used for the spectrophotometric measurement at 530 mμ against water as a control. The

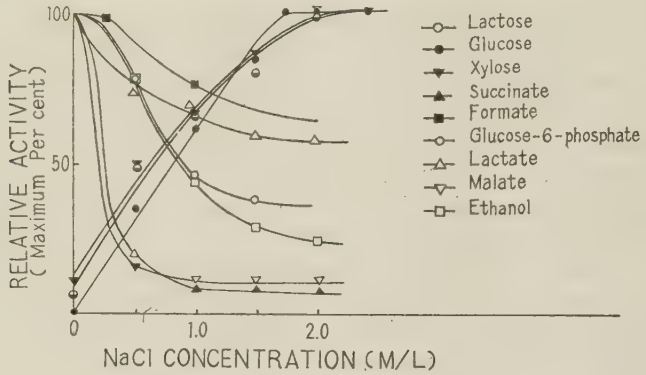

Fig. 1. Effect of NaCl on various dehydrogenase activities. The main tube contained phosphate buffer ($M/4$, pH 7.0) 0.5 ml., substrate ($M/10$) 0.5 ml., toluylene blue (10^{-3} M) 0.5 ml. and NaCl (5 M). The side arm contained 0.5 ml. of enzyme solution. The total volume was 5.0 ml. with water. Methylene blue was also available as hydrogen acceptor for formic, lactic, malic, succinic, glucose-6-phosphate and ethanol dehydrogenases.

same procedure was applied to the reaction mixture in which water was added in place of substrate. The difference in optical densities between main and control experiments was used as a measure of glucose dehydrogenase activity.

Effect of NaCl on Various Dehydrogenases—The enzyme solution showed formic, lactic, malic, succinic, ethanol, glucose-6-phosphate, arabinose, maltose, galactose, xylose, and glucose dehydrogenase activities, but had no ability to oxidize mannose, fructose, acetate and gluconate. Fig. 1 shows the effects of NaCl on these activities. With xylose, maltose and lactose as substrate, dehydrogenase activities were scarcely observed in the absence of added NaCl, but markedly stimulated by the higher concentrations of NaCl, as in the case of glucose dehydrogenase. Glucose dehydrogenase decolorized

toluylene blue but not methylene blue. On the other hand, glucose-6-phosphate dehydrogenase decolorized both dyes, and its activity was weaker in higher concentrations of NaCl than in the lower. In addition, glucose dehydrogenase activity in acetate buffer was found to be the same as in phosphate buffer.

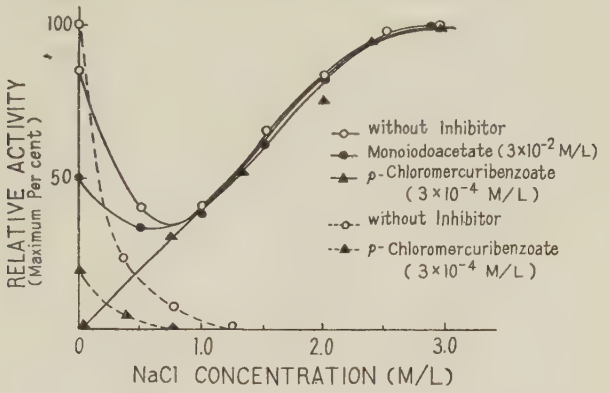

FIG. 2. Effect of NaCl on the dehydrogenase activity with galactose. The activity was measured by Thunberg-technique at pH 7.0.
—— Toluylene blue was used as H-acceptor.
····· Methylene blue was used as H-acceptor.

TABLE I

Decolorization Time in Maximum Activity of Some Dehydrogenases

Substrate	NaCl concentration *M/L.*	Decolorization time *minutes*
Water	0	60
Water	2.0	>180
Lactate	0	26
Malate	0	8
Ethanol	0	2
Succinate	0	7
Glucose*	2.0	16

* The preparation of glucose dehydrogenase was different from others. The experimental conditions were the same as in Fig. 1.

The decolorization time by some dehydrogenases in their maximum activities was shown in Table I. With galactose as substrate, the effect of NaCl on the activity

(Fig. 2) was quite different from that in Fig. 1. Both methylene blue and toluylene blue could be decolorized, and with methylene blue the activity was strongly inhibited by NaCl, and above about 1.0 M NaCl concentration, methylene blue was not decolorized. On the contrary, with toluylene blue the minimum activity was observed at the concentration of 0.75 M NaCl. If *p*-chloromercuribenzoate ($3\times10^{-4}M$/L.) was added to the reaction mixture, an appreciable inhibition was observed with methylene blue in the whole range of the concentrations of NaCl, but with toluylene blue the activity was completely inhibited in the absence of added NaCl but not above about 1 M NaCl concentration by *p*-chloromercuribenzoate.

Some Properties of Glucose Dehydrogenase—Hydrogen Acceptors: Toluylene blue and 2,6-dichlorophenol indophenol were capable of functioning as hydrogen acceptors, but following dyes were incapable: pyocyanine, 2,3,5-triphenyltetrazolium chloride and methylene blue. Oxygen was also inactive as an acceptor.

TABLE II

Effect of Enzyme Concentration on Glucose Dehydrogenase Activity

Enzyme solution *ml.*	Decolorization time *minutes*
1.00	6.5
0.50	12.5
0.25	25
0.17	39

The activity was measured by Thunberg-technique. The main tube contained phosphate buffer (M/4, pH 6.8) 0.5 ml., glucose (M/10) 0.5 ml., toluylene blue ($10^{-3}M$) 0.5 ml., NaCl (5 M/L.) 2.0 ml. and water 1.0 ml. The side arm contained 0.5 ml. of enzyme solution. The final concentration of NaCl was 2.0 M/L.

Effect of Enzyme Concentration: The activity of glucose dehydrogenase was found to be proportional to the concentration of enzyme both in Thunberg-technique with toluylene blue (Table II) and in the spectrophotometric method using indophenol (Fig. 3).

Stability: The enzyme solutions containing 1/40 M phosphate buffer at various pH_s were kept at 3° in a refrigerator. As is shown in Fig. 4, the enzyme at pH 5.5 was more stable than at higher pH_s. The stability of the enzyme with added NaCl at various concentrations was also investigated at 3°. The enzyme solution without added NaCl was almost inactivated within about two days. When solid NaCl was added to 3.0 M in the partially inactivated enzyme solution, the remained activities were, although not recovered, stabilized and kept for days. When frozen, even without the addition of NaCl, more than half of the enzyme activities was retained after a week.

Effect of Various Salts: The effects of salts such as KCl, NaBr, LiCl, Na_2SO_4, sodium citrate, and ethylene glycol were already described by T. Yamada and A. Asano (*5*). As can be seen in Fig. 6, $MgCl_2$ and $CaCl_2$ at lower concentrations than 0.6

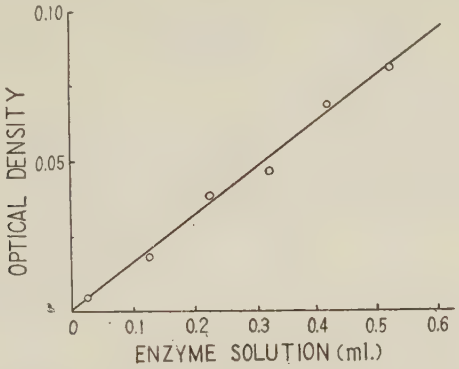

Fig. 3. 2,6-Dichlorophnol indophenol reduction in various enzyme concentrations.
NaCl: 2.0 *M*/L., glucose: 0.1 *M*/L., pH 6.08 phosphate buffer.

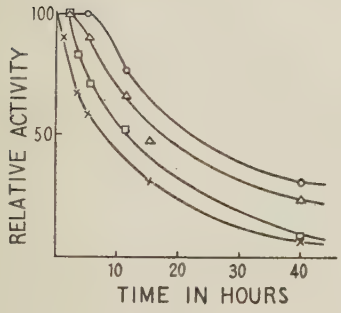

Fig. 4. Stability in Various pH Values in the Absence of Added NaCl. The activities were measured by Thunberg-technique: NaCl 2.0 *M*/L., glucose 0.1 *M*/L., phosphate buffer pH 7.0.
—○— pH 5.5; —△— pH 6.0;
—□— pH 7.0; —×— pH 8.0.

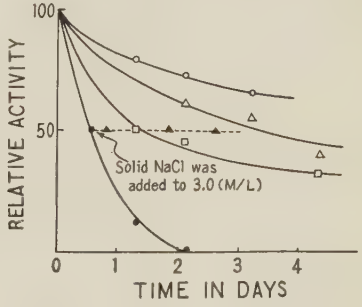

Fig. 5. Stability in Various NaCl concentrations. Assay conditions were the same as in Fig. 4.
—○— 3.0 *M*/L.
—△— 2.0
—□— 0.5
—●— 0
—▲— 3.0

gram cation per liter had a more stimulating effect than NaCl. However, the effect of $MgCl_2$ decreased at higher concentrations than 1.0 gram cation per liter. NH_4Cl

activated the glucose dehydrogenase but its effect was about a half of that of NaCl.

Reaction Rate: That the halophilism of the enzyme cannot be explained by the higher stability at the higher concentration of NaCl was clearly demonstrated by the fact that the reaction rates at various NaCl concentrations proceeded linearly in the first several minutes. 2,6-Dichlorophenol indophenol was more rapidly reduced at 2.1 *M* than at 0.6 *M* NaCl, but the reduction rate at 0.6 *M* NaCl became equal to that at 2.1 *M* NaCl when solid NaCl was added to the reaction mixture to the final concentration of 2.1 *M* NaCl (Fig. 7).

Optimum pH: The optimum pH at 1.0 and 2.0 *M* NaCl was measured (Fig. 8). At 2.0 *M* NaCl the optimum pH was about 6.1–6.2, while it was 5.5 at 1.0 *M* NaCl.

Michaelis Constant: Michaelis constant (Km) was found not to vary with

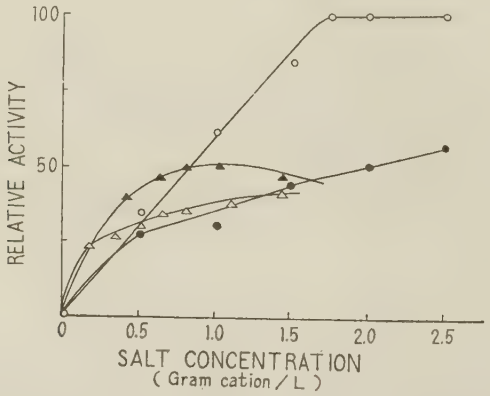

Fig. 6. Effect of $CaCl_2$, $MgCl_2$, NH_4Cl and NaCl on Glucose Dehydrogenase. The assay method was Thunberg-technique.
—○— NaCl; —▲— $NgCl_2$; —△—$CaCl_2$ —●— NH_4Cl.

the NaCl concentration. The values of Km at 2.0 and 1.0 *M* NaCl were the same, *i.e.*, 1.8×10^{-3} *M*.

Inhibition: With indophenol as hydrogen acceptor, effects of various inhibitors on the glucose dehydrogenase were investigated (Table III). Cupric sulfate showed a characteristic inhibition, while neither SH-inhibitors such as monoiodoacetate, *p*-chloromercuribenzoate and $HgCl_2$ nor metal inhibitors such as cyanide and azide inhibited.

DISCUSSION

T. Yamada and A. Asano could obtain nonhalophilic formic dehydrogenase and halophilic glucose dehydrogenase by the exposure

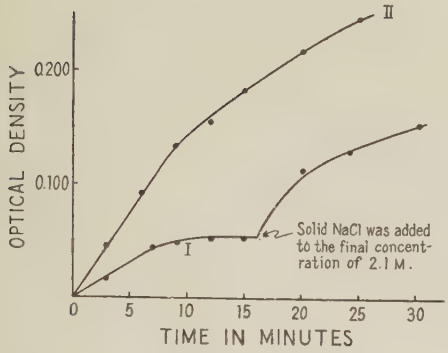

FIG. 7. Reaction rates.

Curve I; 0.6 *M* NaCl concentration for the first 16 minutes.

At the point indicated in the figure, solid NaCl was added to the final concentration of 2.1 *M*/L.

Curve II; 2.1 *M* NaCl concentration.

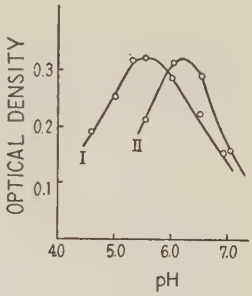

FIG. 8. Optimum pH at 2.0 *M*/L. (courve II) and 1.0 *M*/L. (curve I).

The reaction mixture contained; glucose 0.1 *M*/L., buffer *M*/40, 2,6-dichlorophenol indophenol 7.5 μM. Phosphate buffer was applied for higher pH than 5.5 and acetate buffer for lower pH than 5.5.

The activity change due to the type of the buffers was not observed.

TABLE III

Effect of Inhibitors on Glucose Dehydrogenase

Compounds	Inhibition (%)	
	10^{-4} *M*/L.	10^{-3} *M*/L.
KCN		0
NaN_3		0
Monoiodoacetate		0
p-Chloromercuribenzoate	0	—
$CuSO_4$		100
$ZnCl_2$		50
$FeCl_3$	7	15
$PbBl_2$	0	5
$HgCl_2$		0

2,6-Dichlorophenol indophenol was used as hydrogen acceptor.

of the halophilic bacteria No. 101 to the ultrasonic vibration (*5*). However, we found that these enzymes could be obtained in a clear cellfree state by a simpler method by plasmoptisizing the cells with distilled water. The halophilic alkaline phosphomonoesterase (*2*) was extracted by suspending the cells in water, followed by dialysis against distilled water for three hours, but it was recently found in our laboratory that the alkaline phosphomonoesterase was an extracellular enzyme extrable almost completely from the cells by several washings with 10 per cent NaCl solution. The glucose dehydrogenase, however, was not an extracellular enzyme.

In the oxidation of galactose two pathways may exist in the halophilic bacteria No. 101, one of which is sensitive to *p*-chloromercuribenzoate and the other is not.

It will be doubtless that the halophilic glucose dehydrogenase may take an important part in the mechanism of the halophilism of the cells, but, since the nonhalophilic dehydrogenases could be also extracted from the same cells, the mechanism of the halophilism appears to be quite complex.

It may be concluded from the following facts that glucose may be oxidized without phosphorylation ; 1) the dehydrogenase activities in acetate buffer were the same in phosphate buffer and 2) they were halophilic but glucose-6-phosphate dehydrogenase activities were nonhalophilic.

Although glucose dehydrogenase in the halophilic bacteria No. 107 could not be extracted in a cell-free state by the same procedure as in the case of the halophilic bacteria No. 101, malic, succinc, and glucose-6-phosphate dehydrogenases could be extracted, which were all nonhalophilic enzymes.

Although coenzymes, if any, of the glucose dehydrogenase in the halophilic bacteria No. 101 were not yet investigated, pyridine nucleotides may, possibly, not stimulate the activity since dyes of comparatively high potentials such as toluylene blue or 2,6-dichlorophenol indophenol act as H-acceptors.

As was seen in the halophilic alkaline phosphomonoesterase in our halophiles and glycerol dehydrogenase in *Pseudomonas salinaria* (*7*), the glucose dehydrogenase was also unstable in the absence of added NaCl. This character may be general for the halophilic enzymes.

The glucose dehydrogenase can be stimulated not only by monovalent but bivalent cations such as Mg^{++} and Ca^{++}, and is a typical

halophilic enzyme which has no activity in the absence of salts.

Although $HgCl_2$ has no inhibitory effect, $CuCl_2$ completely inhibit the glucose dehydrogenase at 10^{-3} *M*. Glucose dehydrogenase from the red alga *Iridophycus flaccidum* (*8*) which is probably a flavoprotein is inhibited by $HgCl_2$ at the same concentration but $CuCl_2$ causes no inhibitory effect. The mammalian D-glucose dehydrogenase (*9*) which depends upon the pyridine nucleotides as its coenzymes is inhibited by a low concentration of heavy metals. Glucose dehydrogenase in *P. fluorescens* which depends upon a modified cytochrome transport path (*10*) is strongly inhibited by cyanide. Glucose dehydrogenase in our halophiles was not inhibited by cyanide.

It is an interesting fact that the optimum pH varied with concentration of NaCl.

SUMMARY

1. Among various dehydrogenase activities in the supernatant obtained by bacteriolysis of a halophilic bacterium No. 101, the activities which oxidize glucose, xylose, lactose, maltose, and galactose are halophilic.

2. The glucose dehydrogenase which is characterized by the typical halophilism can catalyze the transfer of hydrogen to dyes of high potentials such as toluylene blue and 2,6-dichlorophenol indophenol but not to methylene blue, pyocyanine, 2,3,5-triphenyltetrazolium chloride and oxygen.

3. The glucose dehydrogenase is rather stable in high salt concentrations but irreversibly loses its activities in the absense of sodium chloride.

4. Not only monovalent cations but also bivalent cations such as Mg^{++} and Ca^{++} activate the glucose dehydrogenase. However, Cu^{++} inhibit completely the activities.

5. Optimum pH varies with the concentration sodium chloride, but Michaelis constant does not.

The author wishes to thank Prof. Fujio Egami for his helpful encouragement and his useful discussions.

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MICROBIOLOGICAL DEGRADATION OF BILE ACIDS

X. A NEW DEGRADATION PRODUCT OF CHOLIC ACID BY STREPTOMYCES GELATICUS 1164

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(Received for publication, October 5, 1956)

In a previous paper (*1*) of this series, it has been demonstrated that from *Streptomyces gelaticus* 1164 culture containing cholic acid and glucose as the carbon sources, several oxidation products of cholic acid (Scheme, I) such as 3α,7α-dihydroxy-12-ketocholanic acid (Scheme, III), 7α,12α-dihydroxy-3-ketocholanic acid, 7α-hydroxy-3,12-diketocholanic acid and 3,12-diketo-$\Delta^{4,6}$-choladienic acid were isolated as their methyl esters.

The above-obtained methyl 3,12-diketo-$\Delta^{4,6}$-choladienate (Scheme, II′) is considered to be derived artificially from its precursor acid II (Scheme), 7α-hydroxy-3,12-diketo-Δ^{4}-cholenic acid, by methanolic sulfuric acid treatment in the isolation process, because 7α-hydroxy-3,12-diketo-Δ^{4}-bisnorcholenic acid (Scheme, IV) which was isolated previously (*2–3*) from a medium containing no glucose was converted to methyl 3,12-diketo-$\Delta^{4,6}$-bisnorcholadienate (Scheme, IV′) by the same treatment (*3*).

In the present study, the methyl ester of 3α,7α-dihydroxy-12-ketobisnorcholanic acid (Scheme, V) and methyl esters II′ and IV′ were isolated from the glucose-containing media of *S. gelaticus* 1164 after a more protracted time of incubation by the same treatments reported before (*1*). The parent acids of these esters II′ and IV′ are probably the acids II and IV respectively as described in a previous paper (*1*).

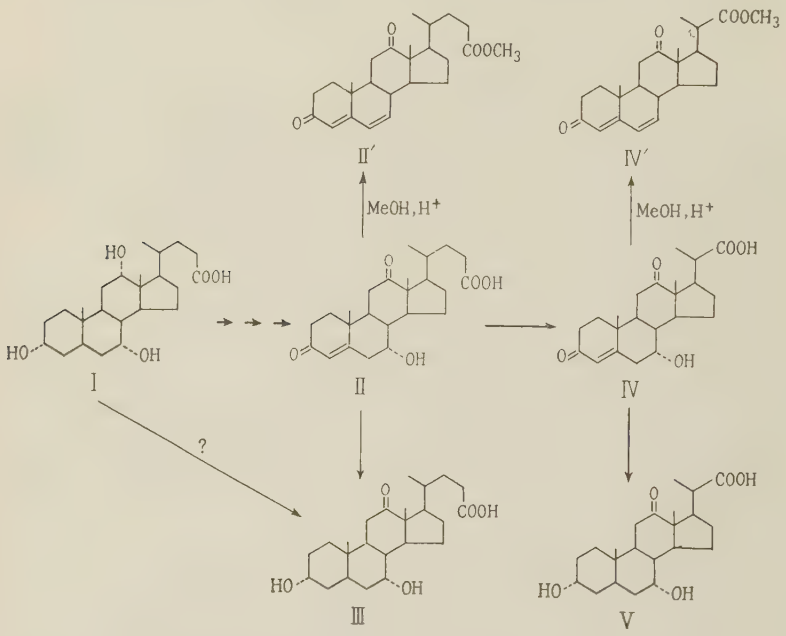
The isolation of the esters II′ and IV′ suggests that a Δ^{4}-3-ketone structure is necessary for the oxidative splitting of the steroid side chain by microbes. In support of this suggestion it may be recalled that Peterson *et al.* (*4*) succeeded in degrading progesterone containing a

Δ^4-3-ketone group to 4-androstene-3,17-dione *etc.* by several microorganisms.

Assuming that a Δ^4-3-ketone structure is necessary for the oxidation of a steroid side chain, acid V may be a reduction product of acid IV.

In the case of the short time incubation (*1*), acid III was one of the most abundant products and was at first presumed to be derived directly from cholic acid (I). The isolation of acid V, however, has led to a reconsideration of the above presumption.

Now it is quite possible that acid III may be derived from acid II by the same manner as pointed out on the formation of acid V.



Therefore a possible pathway for the formation of the newly isolated compound V and the previously isolated compound III may be written as shown in the scheme.

The acids III and V have not yet been isolated from a medium containing no glucose, so it is quite likely that glucose may supply during its degradation a large amount of reduced coenzyme for the hydrogenation of the Δ^4-3-ketone grouping.

EXPERIMENTAL

Cultivation—According to the same method as described in a previous paper (*1*), six grams of sodium cholate were incubated with *S. gelaticus* 1164 for 10 days. The pH of the culture filtrate dropped to 5.0 from the original pH 7.0 and the acid precipitation test and Pettenkofer's test of the media were completely negative at the end of the incubation.

Extraction and Esterification—The contents of the flasks were combined, the mycellium removed by filtration, and adjusted to about pH 6.8 with dilute sodium carbonate solution. The neutralized solution (2,000 ml) was concentrated under reduced pressure to about 240 ml. The temperature was kept below 40° during this operation. The concentrated culture filtrate was acidified (about pH 4.0) with dilute hydrochloric acid and extracted twice with 200 ml. of ether. The combined ether extracts were concentrated to about 150 ml. at below 50° and gave 160 mg. of needles which separated out on standing. Attempts to identify these crystals are not yet successful.

The ether filtrate, filtered off these crystals, was evaporated to dryness and afforded 1.730 g. of an uncrystallizable yellow gum. The resulting gum was esterified by refluxing with 35 ml. of methanol containing 1.8 g. of concentrated sulfuric acid for five hours. The reaction mixture was poured into about 350 ml. of water and left standing overnight. The resultant precipitate was collected and dissolved in 40 ml. of ether. The yellowish ether solution was washed with 1 per cent aqueous ammonia solution and then with water, and dried with anhydrous sodium sulfate. The dried ether solution was evaporated to dryness leaving 1.004 g. of a yellowish oily mass.

Chromatographic Separation of Methyl 3α,7α-Dihydroxy-12-ketobisnorcholanate—The above-obtained oily mass was dissolved in 20 ml. of ether and the ether solution was poured onto an active alumina (30 g. of Wako, 200 mesh) column of dimensions 205 × 14 mm. The effuent was colorless and gave no residue after evaporation of ether. Only two (fractions 4 & 5) out of eleven elutions (fractions 1 to 11) with 10 ml. of pure ether each afforded a small amount of crystals with m.p. 170–173°, which are probably methyl 3,12-diketo-$\Delta^{4,6}$-choladienate from the consideration of the data described in a previous paper (*1*). The succeeding two elutions (fractions 12 & 13) with 10 ml. of ether each and the fourteenth elution with 25 ml. of ether—methanol (4:1) yielded no residue after evaporation of the solvents. Also two elutions (fractions 16 & 17) with 25 ml. of ether—methanol (1:1) and the eighteenth elution with 50 ml. of methanol yielded a trace of non-crystalline material. Elutant fraction 15 with 25 ml. of ether—methanol (4:1) yielded a crystalline residue. Recrystallization of this crystalline mass from ethyl acetate—petroleum ether afforded 40 mg. of needles sintering at 195° and melting at 201–203°. The ultraviolet absorption spectrum showed no characteristic absorption in the region of 220 to 330 mμ and the infrared spectrum showed the following absorption bands (KBr disk): hydroxyl, 2.94 μ; ester and ketone, 5.74 and 5.91 μ.

Anal. Calcd. for $C_{23}H_{35}O_5$: C, 70.37; H, 9.24.
Found: C, 69.70; H, 9.04.

This ester was confirmed to be identical with methyl 3α,7α-dihydroxy-12-keto-bisnorcholanate by converting to its acetate as follows.

Methyl 3α,7α-Diacetoxy-12-ketobisnorcholanate—

A) From the above-obtained ester with m.p. 201–203°: Twenty milligrams of this ester, isolated from the incubation mixture, were refluxed with 2 ml. of acetic anhydride for two hours. The resulting solution was evaporated in vacuo to dryness and afforded 20 mg. of needles. Crystallization from methanol gave 14 mg. of prismatic needles with m.p. 216–218°. Identity of this acetate with an authentic methyl 3α,7α-diacetoxy-12-ketobisnorcholanate was demonstrated by a mixed melting point determination and infrared comparison.

Anal. Calcd. for $C_{27}H_{40}O_7$: C, 68. 04; H, 8.46.
Found: C, 68. 62; H, 8.36.

B) From methyl bisnorcholate 3,7-diacetate: A solution of 15 mg. of potassium chromate in one drop of water was added to a solution of 20 mg. of methyl 3α,7α-diacetoxy-12α-hydroxybisnorcholanate with m.p. 180–182°, described in a previous paper (*5*) in 0.3 ml. of glacial acetic acid and the resulting mixture was allowed to standing at room temperature (about 29°) for 20 hours and then diluted with 1 ml. of water. The crystalline precipitate which separated out on standing, was collected, washed with water and dried. Crystallization from methanol afforded 15 mg. of prismatic needles. The melting point of the substance was 218–220°; Takamori (*6*) reported that this substance with m.p. 220° was obtained by chromic anhydride oxidation of methyl bisnorcholate 3,7-diacetate. The infrared spectrum showed the following absorption bands (KBr disk): ester and ketone, 5.77 and 5.88 μ; acetate 8.01 and 8.09 μ.

Anal. Calcd. for $C_{27}H_{40}O_7$: C, 68.04; H, 8.46.
Found: C, 68.46; H, 8.24.

Isolation of Methyl 3,12-Diketo-$\Delta^{4,6}$-choladienate and Methyl 3,12-Diketo-$\Delta^{4,6}$-bisnorcholadienate—Since the yield of the various intermediates seems to be dependent on the incubation periods, in this experiment 1.5 g. of sodium cholate was incubated with *S. gelaticus* 1164 for 7 days in the same medium as described in a previous paper (*1*). After the incubation, the filtrate was concentrated, acidified and extracted with ether. The ether extracts were evaporated to dryness, esterified with methanol and sulfuric acid and then chromatographed on an alumina column. The yellow-colored elutants in the beginning of the elutions with ether were re-chromatographed on an alumina column. Two separate fractions were obtained by elution with ether. Yellow crystals with m.p. 170–172° were obtained from a faster running fraction. Since these crystals gave no depression of melting point with an authentic sample, they may be methyl 3,12-diketo-$\Delta^{4,6}$-choladienate (*1*) although further identification was impossible because of a poor yield.

Pale yellow crystals with m.p. 175–177° were obtained from another elution with ether. These crystals were further recrystallized from ethyl acetate—petroleum ether

and gave 10 mg. of prismatic needles with m.p. 177–179°. The infrared absorption spectrum showed the following absorption bands (KBr disk): ester and ketone, 5.78 and 5.84 μ; $\Delta^{4,6}$-3-ketone, 6.04, 6.185 and 6.31 μ. The ester was still not sufficiently pure to give satisfactory microcombustion data, but it melted at 178–182° when mixed with the known sample (*7*) with m.p. 181–183° and infrared analysis confirmed the identity of this ester.

Anal. Calcd. for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16.
Found: C, 73.87; H, 7.97.

SUMMARY

1. A new degradation product of cholic acid by *S. gelaticus* 1164 was isolated as its methyl ester from a medium containing sodium cholate and glucose as the carbon sources.

2. The ester was confirmed to be methyl 3α,7α-dihydroxy-12-ketobisnorcholanate by its conversion to the known acetyl derivative.

3. 3,12-Diketo-$\Delta^{4,6}$-bisnorcholadienic acid, corresponding to a dehydration product of 7α-hydroxy-3,12-diketo-Δ^4-bisnorcholenic acid which was isolated previously from a medium containing cholic acid as the sole source of carbon, was also isolated as its methyl ester from a medium containing sodium cholate and glucose as the carbon sources.

4. A possible pathway from cholic acid to the formation of 3α, 7α-dihydroxy-12-ketobisnorcholanic acid isolated this time and 3α, 7α-dihydroxy-12-ketocholanic acid previously isolated (*1*) was discussed.

The authors express their hearty thanks to Prof. T. Shimizu and Prof. S. Mizuhara for their interest and advice in this work, and also to Mr. M. Seki of Department of Chemistry, Faculty of Science, Nagoya University for his help in measuring the infrared absorption spectra.

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METABOLIC STUDIES OF BILE ACIDS

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(Received for publication, October 30, 1956)

Previous *in vivo* studies (*1*, *2*, *3*) have revealed that bile acids, such as cholic or lithocholic acid can be biosynthesized from cholesterol. The pathway through which cholesterol is metabolized to the bile acid, however, still remains obscure.

One of the probable metabolites of cholesterol, Δ^5-3-β-hydroxycholenic acid has recently become easily available by dehydration of hydexoycholic acid (*4*). We are now studying its significance in biosynthesis of the bile acid.

In this paper will be reported the metabolic study of Δ^4-3-ketocholenic acid, derived from this acid, which has a structure analogous to progesterone. *In vitro* studies of some other 3-keto bile acids were also undertaken, compared with the metabolism of progresterone.

EXPERIMENTAL

Adult rats were killed by decapitation, their livers were removed as rapidly as possible and homogenized, being chilled in ice-cold water. The homogenate, so prepared, was then diluted with Krebs-Ringer phosphate solution (pH 7.4) to a final concentration of 2 per cent. The methyl ester of each bile acid (1.5 μM) was dissolved in 0.3 ml. propylene glycol and incubated with 4 ml. of the homogenate for 1 hour at 38° by means of Warburg's apparatus, the gas phase being O_2 (unless otherwise stated).

The following methyl esters of 3-keto bile acids were subjected to investigation: Δ^4-3-ketocholenate (m.p. 131°), 3-ketocholanate (m.p. 117–118°), 3-ketoallocholanate (m.p. 114°). Soon after the incubation period was over, 4 ml. of ethanol and 0.5 ml. of 5 per cent HCl were added to each incubation flask, and the mixture was heated for 10 minutes on a boiling water-bath. The cooled mixture was diluted with 30 ml. water, and extracted twice with 20 ml. of ether. The combined ether layer was washed with 10 ml. saturated NaCl solution, and distilled off to dryness. The residue was dissolved in 1 ml. absolute ethanol, and the residual C_3-keto bile acid was determined photometrically by the modified method of Miyashita (*5*) for determination of de-

hydrocholic acid.

Each experiment was conducted in three parts: (1) Incubation experiments were carried out in the manner described above. (2) Recovery controls: Each bile acid was added to the homogenate, whose enzymatic activity was beforehand destroyed by heating after addition of ethanol and HCl. (3) Blank tests: 0.3 ml. propylene glycol without bile acid was incubated just like the experiment (1).

The values of the residual 3-keto bile acids in the incubation mixture are expressed in per cent of those of recovery controls. It was found that the bile acids added to the homogenate combined with the tissue proteins so fast, that some part of them remained unextracted even in the recovery controls, where they were recovered in 60± 10 per cent yield.

RESULTS

Fig. 1 shows the enzymatic activity of rat liver homogenate on Δ^4-3-ketocholenate and how the activity can be influenced by addition of some reagents. It was found that about 40 per cent of the added Δ^5-3-ketocholenate disappeared in the incubation medium.

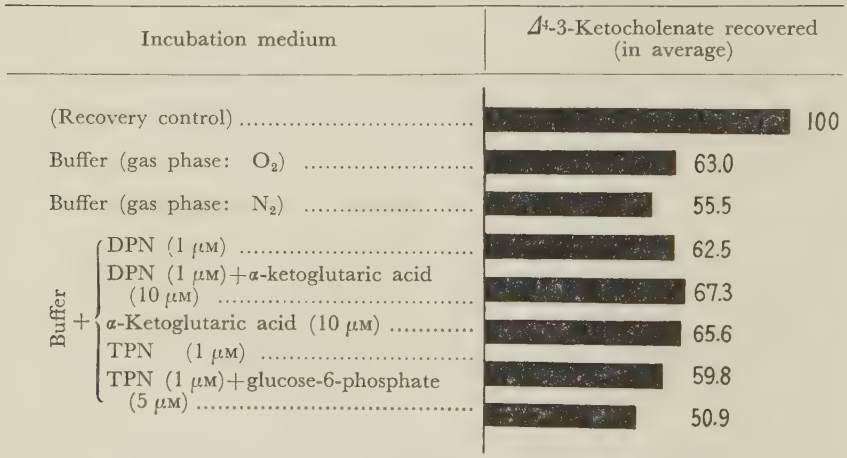

FIG. 1. Incubation of Δ^4-3-ketocholenate with rat liver homogenate. The values of Δ^4-3-ketocholenate in the incubation experiments are expressed in per cent of those of the recovery controls.

Under anaerobic conditions, in which the gas phase of the incubation flask was replaced with N_2, the enzymatic activity was slightly accelerated.

Since it has been reported by Taylor (*6*) that the metabolism of progesterone was accelerated by addition of DPN in an *in vitro* study, the effect of this co-factor upon the metablism of Δ^4-3-ketocholenate was examined. No significant acceleration of the enzymatic activity could be demonstrated either by adding 1 μM DPN to the incubation media or by simultaneous addition of α-ketoglutaric acid (10 μM).

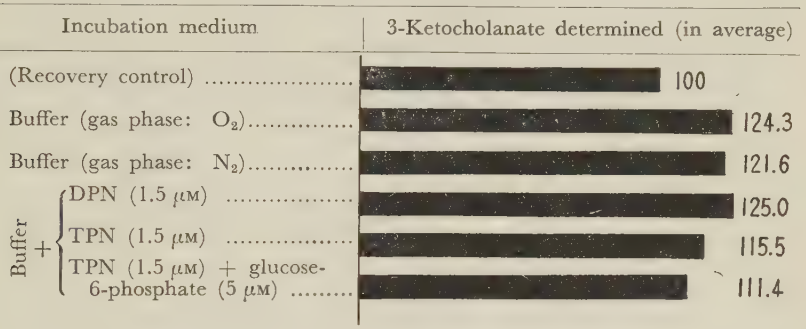

Fig. 2. Incubation of 3-ketocholanate with rat liver homogenate. The values of 3-ketocholanate in the incubation experiments are expressed in per cent of those of the recovery controls.

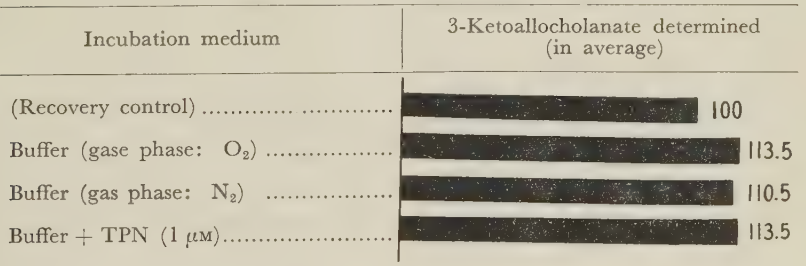

Fig. 3. Incubation of 3-ketoallocholanate with rat liver homogenate. The values of 3-ketoallocholanate in the incubation experiments are expressed in per cent of those of the recovery controls.

On the other hand, the metabolic rate of Δ^4-3-ketocholenate was increased by addition of TPN (1 μM), and simultaneous addition of glucose-6-phosphate (5 μM) caused a remarkable acceleration of the process, namely, about 50 per cent of the bile acid disappeared.

As shown in Fig. 2, 3-ketocholanate, contrary to Δ^4-3-ketocholenate, was recovered in larger amount than was added in the incubation

media. While neither anaerobic condition nor addition of DPN (1.5 μM) gave any effect in this experiment, addition of TPN (1.5 μM) or TPN (1.5 μM) plus glucose-6-phosphate (5 μM) caused a slight depression of the increased recovery of the added bile acid.

In the incubation experiment of 3-ketoallocholanate analogous results were obtained (Fig. 3). However, the increased recovery of the bile acid was somewhat depressed in the anaerobic condition, but not influenced by addition of TPN (1 μM).

Since the calibration curve of Δ^4-3-ketocholenate in the method of determination employed here, shows a coefficient about three times as

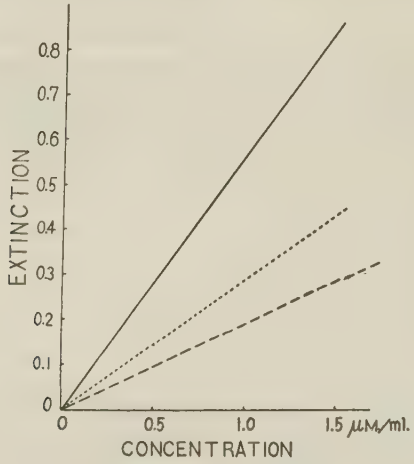

Fig. 4. Calibration curves of 3-ketobile acids.
—— Δ^4-3-Ketocholenate. --- 3-Ketocholanate.
····· 3-Ketoallocholanate.

high as that of 3-ketocholanate, and about twice as high as that of 3-ketoallocholanate (Fig. 4), such increased recovery of 3-ketocholanate and 3-ketoallocholanate in the incubation experiments might be due to the production of Δ^4-3-ketocholenate by dehydration of these keto bile acids.

Accordingly another series of experiments was carried out to prove this possibility. Since Δ^4-3-ketocholenate exhibits a maximum absorption at 250 mμ (7) (Fig. 5), the optical density at this wave length was determined to detect the appearance of Δ^4-3-ketocholenate in the incubation media.

These saturated 3-keto bile acids as well as Δ^4-3-ketocholenate were incubated in the same manner as described above. To the reaction mixture was added 5 ml. ethanol and the mixture was heated for 10 minutes on a boiling water-bath, diluted with ethanol, after cooling, to the final volume of 30 ml., and filtered. The filtrate was then subjected to the spectrophotometric measurement.

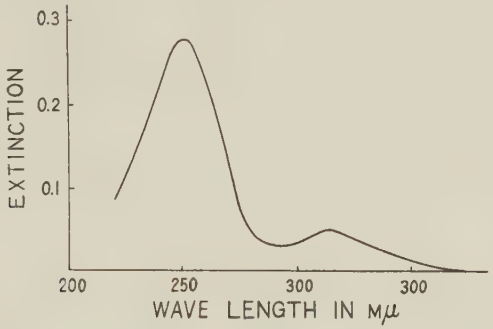

Fig. 5. Ultraviolet absorption spectrum of Me-Δ^4-3-ketocholenate in 99 per cent ethanol (10 μg./ml.). $\varepsilon_{250}=2.8\times10^4$.

Bile acid incubated	Δ^4-3-Ketocholenate determined (in average)
(Recovery control of Δ^4-3-ketocholenate)	100
Δ^4-3-Ketocholenate	72.8
3-Ketocholanate	1.7
3-Ketoallocholanate	2.3

Fig. 6. Incubation of 3-keto bile acids (spectrophotometric determination). The values of Δ^4-3-ketocholenate in the incubation experiments are expressed in per cent of those of the recovery controls.

As shown in Fig. 6, it is obviously indicated that the conjugated ketone group of the ketocholenate, which gave a maximum absorption at 250 mμ, was destroyed to a measurable extent by the enzyme system of rat liver homogenate, just as the preceeding experiments (Fig. 1) predict. The oxidative dehydration of 3-ketocholanate or 3-ketoallocholanate to Δ^4-3-ketocholenate, however, could not be demonstrated.

DISCUSSION

The evidence presented here shows that Δ^4-3-ketocholenate undergoes metabolic degradation by an enzyme system in the rat liver homogenate.

The process seems to be a metabolic destruction of the conjugated double bond system of the bile acid molecule, that gives a maximum absorption at 250 mμ. From the finding that the activity of the enzyme system involved, was increased by TPN, but not by DPN, it appears that TPN is a specific co-factor catalyzing the process.

The fact that the effect of TPN was accelerated in the presence of glucose-6-phosphate clearly shows that the process is catalyzed by the reduced TPN, which is expected to appear in the course of glucose phosphate oxidation through the Warburg-Dickens pathway.

According to the current view (*8*), steroid hormones such as testosterone, progesterone, desoxycorticosterone, and the like undergo quite the same sequence of metabolic reactions, namely the saturation of the conjugated double bond system in ring A followed by reduction of C_3-ketone to C_3-alcohol. Consequently, it seems probable that Δ^4-3-ketocholenate is metabolized, in an analogus manner, to 3-ketocholanate by saturation of the double bond and then to 3-hydroxycholanate (lithocholate).

But the present work failed to demonstrate enzymatic reduction of 3-ketocholanate to lithocholate, and, on the contrary, 3-ketocholanate was recovered in increased amounts from the incubation media. The reversed process explicable of this finding, *i.e.* the possibility that Δ^4-3-ketocholenate is produced by dehydration from 3-ketocholanate or 3-ketoallocholanate, however, could not be demonstrated here.

It has been shown by Samuels *et al.* (*9*) that pregnenolone is converted enzymatically to progesterone, and since Δ^5-3-β-hydroxycholenate has a structure analogous to pregnenolone, it might be oxidized in the similar way, though not yet realized, to Δ^4-3-ketocholenate, which in turn may be expected to give rise to the bile acid.

SUMMARY

Δ^4-3-Ketocholenate, 3-ketocholanate and 3-ketoallocholanate were incubated with rat liver homogenate, and the experimental results were as follows:

1. Rat liver contains an enzyme system capable of catalyzing the

reductive process of Δ^4-3-ketocholenate.

2. The conjugated double bond system of the Δ^4-3-ketocholenate molecule, which gives a maximum absorption at 250 mμ, on incubation was destroyed to some extent.

3. Such a metabolic process was accelerated by TPN, especially in the presence of glucose-6-phosphate, while no acceleration was demonstrated by addition of DPN.

4. On incubation of 3-ketocholanate and 3-ketoallocholanate, it was unable to demonstrate the enzymatic reduction of their C_3-ketone group, and on the contrary, they were recovered in increased amounts from the incubation mixture.

The present work was aided by the Grant in Aid for Scientific Research from the Ministry of Education, for which our gratitude is due.

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THE STREAMING TRANSPARENCY OF THE ERYTHROCYTE SUSPENSION

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When the human erythrocytes which are biconcave discoid, 7 μ in diameter and 2 μ in thickness, are suspended in the physiological saline solution and stirred, the transmitted light through the erythrocyte suspension is more intensive in the flowing state than in the resting. The author (*1*) called this special property the " streaming transparency " of erythrocyte suspension and devised a method for determining the " degree of streaming transparency " which is expressed quantitatively by the difference in the intensity of transmitted light between the flowing erythrocyte suspension and the resting one.

In this paper some important factors responsible for the streaming transparency are discussed.

EXPERIMENTAL AND RESULTS

1. The Orientation of Erythrocytes in the Flowing Suspension

The shape of erythrocyte seems to be essential for the streaming transparency, because this is only positive with the biconcave discoid erythrocyte but not with the spheric one.

In the resting erythrocyte suspension, erythrocytes lie at random directions (Fig. 1 A). When the suspension flows along the direction of the arrow, most erythrocytes are oriented parallel to this direction (Fig. 1 B). If light is projected from the direction of the arrow, the number of erythrocytes perpendicular to the projected light increases in the flowing suspension, compared with that in the resting in which red blood cells remain at random directions. The erythrocyte perpendicular to the projected light reflects and scatters the light at minimum, compared with the erythrocyte in any other position and thus the light transmitted through the erythrocyte becomes maximum in intensity. Therefore, the large transparency of the flowing erythrocyte suspension is well attributable to larger amount of light transmitted through ery-

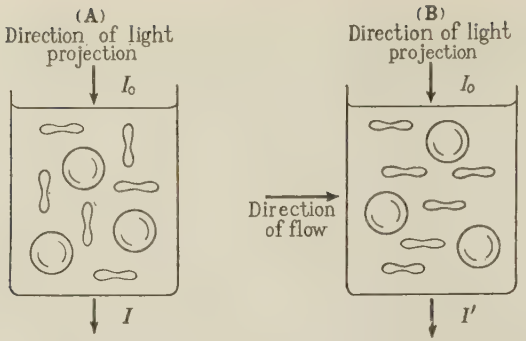

Fig. 1. The orientation of discoid erythrocytes suspended in flowing medium and in resting medium.

A. Resting erythrocyte suspension.

B. Flowing erythrocyte suspension

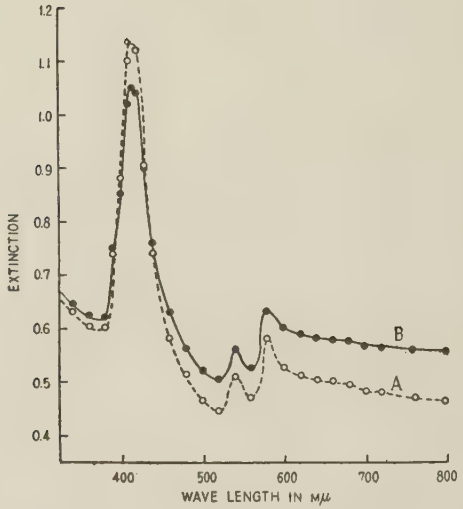

Fig. 2. Light absorption curves of 0.05 per cent human erythrocyte suspension in physiological saline solution.

A. Flowing erythrocyte suspension.

B. Resting erythrocyte suspension.

throcytes. In order to confirm this hypothesis, light absorption curves of both flowing and resting erythrocyte suspensions were studied. In Fig. 2, the extinction of the suspension near the wave length of 415 mμ is larger in the flowing state than in the resting, while at other wave length, especially above 600 mμ, the extinction is smaller in the flowing state than in the resting. The fact is well explained from the following consideration. Since the light projected into erythrocytes in the suspension is more intensive in the flowing state than in the resting, the light is influenced by the hemoglobin in the erythrocyte. Hemoglobin absorbs completely the special light of the wave length 415 mμ. Therefore, the extinction at 415 mμ shows the intensity of the light projected into the erythrocytes. As shown in Fig. 2, the fact that the extinction of the erythrocyte suspension at 415 mμ is larger in the flowing state than in the resting, means that more light projects into the erythrocytes in the former state than in the latter.

On the other hand, the light which can not be absorbed by the hemoglobin in the erythrocyte, especially light of long wave length above 600 mμ, can pass through the erythrocytes freely. Thus, the extinction becomes smaller with increasing amount of the light projected into the erythrocytes. As shown in Fig. 2, the extinction of the erythrocyte suspension above 600 mμ is remarkably smaller in the flowing state than in the resting, showing that more light passes through the erythrocytes in the flowing state than in the resting.

2. Theoretical Consideration of the Streaming Transparency

To the dilute erythrocyte suspension of the concentration less than 0.1 per cent by volume, also, Lambert-Beer's law is applicable approximately (*2*). When an erythrocyte suspension, the concentration of which is c, is placed in the glass vessel of depth r (Fig. 1 A) and is projected from the direction of the arrow by the light of intensity I_0, the intensity I of the light transmitted through the erythrocyte suspension is according to Lambert-Beer's law

$$I = I_0 e^{-hcr} \quad \text{..... (1)}$$

where h is the extinction coefficient of the suspension.

If the erythrocyte suspension flows along the direction of the arrow (Fig. 1 B) and the light of intensity I_0 is cast upon it, the transmitted light I' is obtained in the same way

$$I' = I_0 e^{-h'cr} \quad \text{..... (2)}$$

From the experimental result,

$$I' > I \quad \text{..... (3)}$$

Therefore,

$$h' < h \quad \text{..... (4)}$$

Namely, it is considered that the extinction coefficient of the erythrocyte suspension is changed from h to h' by the alteration of orientation of discoid erythrocytes due to the flow of the suspension.

If k is expressed as follow,

$$h'/h = k \quad \text{..... (5)}$$

k denotes the sphericity of the suspended erythrocyte, and in the case of spheres $k=1$, while in the case of non-spheres $k<1$. From equation 5 and 2,

$$I' = I_0 e^{-khcr} \quad \text{..... (6)}$$

Since the degree of streaming transparency S is defined as the difference in the intensity of transmitted light between the flowing erythrocyte suspension and the resting one, the following equation is obtained, where a is a constant

$$S = aI_0(e^{-khcr} - e^{-hcr}) \quad \text{..... (7)}$$

Now, in the molecule disperse system or in the colloid disperse system, light can not pass through individual molecule or colloid particle and therefore the number of the dispersed particles in an unit volume of solution can be directly used as the optical concentration of the disperse system, while this is not the case in the erythrocyte suspension since light can pass through the interior of erythrocytes. Thus, as the concentration of the coarse disperse system c should be used the product ni

$$c = ni \quad \text{..... (8)}$$

where n means the number of the erythrocyte contained in an unit volume of the suspension and i denotes the optical density of the erythrocyte.

Then, the equation 7 is written as follows

$$S = aI_0(e^{-khnir} - e^{-hnir}) \quad \text{..... (9)}$$

where a, I_0 and h are constants and k, n, i and r are variants.

The function S shown by the equation 9 has a maximum value and a point of inflexion with regard to n, i or r and the abscissa of the point of inflexion (n_i) is two times the abscissa of the maximum value

(n_{max}) (Fig. 3).

The function S is also an exponential function of k and k ranges from 1 to 0. If $k=1$, $S=0$ and if $k=0$, $S=a\ I_0\ (1-e^{-hnir})$ (Fig. 4).

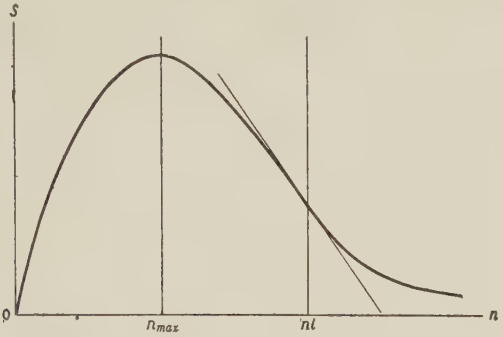

Fig. 3. Degree of streaming transparency (S)—concentration of erythrocyte (n) curve plotted theoretically by equation (9).

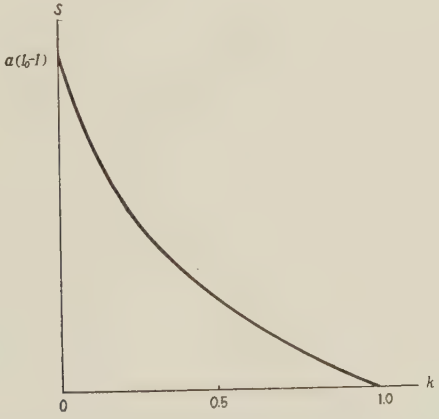

Fig. 4. Degree of streaming transparency (S)—sphericity of erythrocyte (k) curve plotted theoretically by equation (9).

3. Experimental Proof of the Theory of the Streaming Transparency

In order to confirm experimentally the theoretical relationships above mentioned, the correlation between the streaming transparency S and the concentration of erythrocytes c was first investigated. The

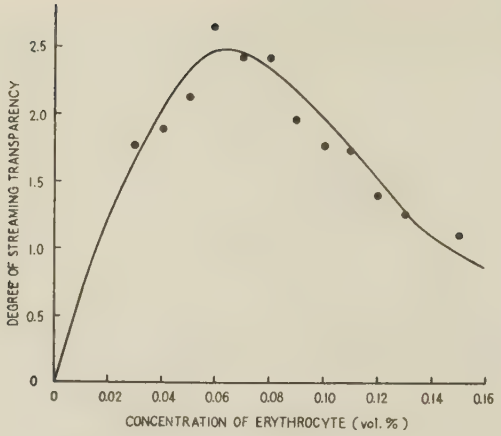

FIG. 5. Relation between the degree of streaming transparency and the concentration of human erythrocyte suspended in physiological saline solution.

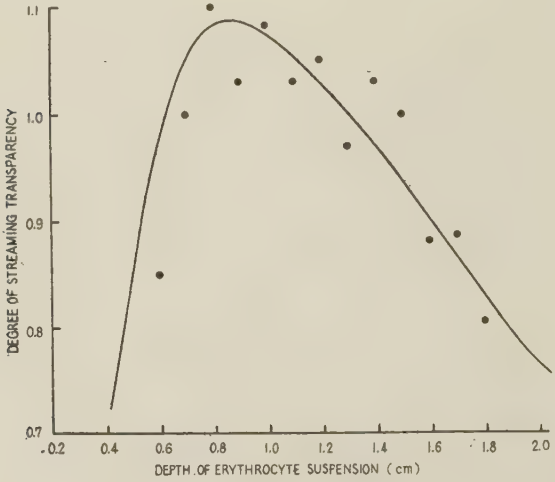

FIG. 6. Relation between the degree of streaming transparency and the depth of 0.1 per cent human erythrocyte suspension in physiological saline solution.

degree of streaming transparency was measured by the author's method. As shown in Fig. 5, a curve with a maximum value and a point of in-

flexion was obtained, similar to Fig. 3.

In the experiment to find out the correlation between the streaming transparency S and the suspension depth r, the same result was obtained. Namely the obtained S-r curve with a maximum value as shown in Fig. 6 was similar to the theoretical curve (Fig. 3).

As for the relation of the optical density i of erythrocytes to the streaming transparency S, there exists the correlation as shown in Fig. 3 theoretically. Therefore, if i is large, the value of S is expressed in the right branch of the curve in Fig. 3 and, with increasing value of i, the streaming transparency should be decreased.

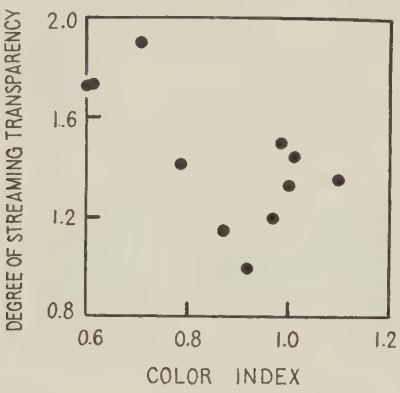

Fig. 7. Correlation between the color index and the degree of streaming transparency of 0.1 per cent human erythrocyte suspension in physiological saline solution.

Correlation coefficient between them is -0.66 ± 0.18.

In this point, the optical density of the erythrocytes i is related to the concentration of the material contained in erythrocytes as well as to the structure of erythrocyte membranes. Since hemoglobin is chief component of interior substance of erythrocytes, the former can be represented by color index which means the hemoglobin content of one erythrocyte. Thus the streaming transparency of the erythrocyte suspension is investigated upon patients of various anemias with different color index. The correlation between the color index and the streaming transparency (Fig. 7) shows the negative correlation, the correlation coefficient being -0.66 ± 0.18, which is expected theoretically.

Next, in an attempt to change the optical density of the erythrocyte

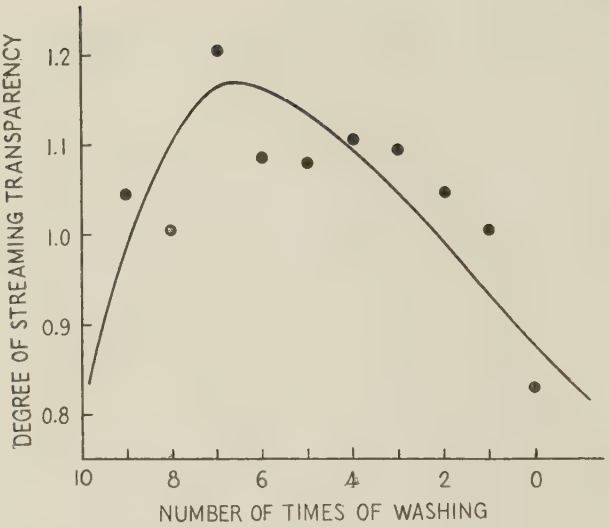

Fig. 8. Relation between the number of times of washing and the degree of streaming transparency of 0.1 per cent washed human erythrocyte suspension in physiological saline solution.

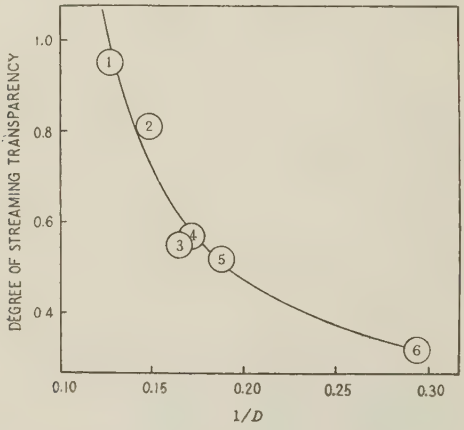

Fig. 9. Relation between the diameter of mammalian erythrocyte and the degree of streaming transparency of 0.1 per cent erythrocyte suspension in physiological saline solution.

1. Human; 2. Rabbit; 3. Pig; 4. Bovine; 5. Horse; 6. Goat

membrane, erythrocytes of human being were washed repeatedly with the physiological saline solution according to Lovelock (*3*). With washings, the erythrocyte membrane became thin and the optical density of the membrane decreased. The streaming transparency of such erythrocyte suspensions were measured and its relation to the times of washing is shown in Fig. 8 which is similar to the theoretical curve of Fig. 3.

The streaming transparency S is also an exponential function of sphericity k of the erythrocyte according to the equation 9 (Fig. 4). In order to confirm the relation, the streaming transparency and diameter D of different mammalian erythrocytes were measured. Mammalian erythrocytes are discoid shaped and when the thickness are assumed to be almost of similar size, $1/D$ is considered to be the sphericity. And when S is plotted against $1/D$, an exponential curve is obtained as shown in Fig. 9 which is similar to the theoretical curve of Fig. 4.

SUMMARY

1. When human erythrocytes are suspended in the physiological saline solution in the concentration less than 0.1 per cent by volume, filled in a glass vessel and the suspension is made flow by rotation, erythrocytes are orientated so as to take their disk surface parallel to the direction of flow.

2. Therefore, when light perpendicular to that direction is passed through the erythrocyte suspension, the transmitted intensity comes out larger when the suspension is placed in the flowing state than in the resting. Herein exists the cause of the streaming transparency.

3. When the suspension which contains n erythrocytes of optical density i per an unit volume is placed in a vessel of depth r, and the light intensity is I_0, the streaming transparency S is expressed theoretically by the following equation:

$$S = aI_0(e^{-khnir} - e^{-hnir})$$

where a is a constant, h is the extinction coefficient of the suspension and k means the sphericity of the erythrocyte. The theoretical equation is also confirmed experimentally.

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ENZYMATIC PHOSPHORYLATION OF ACETATE BY MYCOBACTERIUM TUBERCULOSIS AVIUM

Sirs:

It is well known that acetokinase which appears to be restricted to bacteria catalyzes the formation of acetyl-phosphate (acetyl-P) and adenosine diphosphate (ADP) from acetate and adenosine triphosphate (ATP) (Reaction 1):

$$\text{ATP} + \text{Acetate} \rightleftharpoons \text{Acetyl-P} + \text{ADP}. \qquad (1)$$

The enzymatic phosphorylation of acetate was studied by Lipmann (*1*), Stadtman and Barker (*2*), and Kaplan and Lipmann (*3*). Purified preparations of acetokinase were obtained by Rose *et al.* (*4*), both from *Streptococcus haemolyticus* and from *Escherichia coli*. Partially purified preparations of acetokinase have now been obtained from *Mycobacterium tuberculosis avium*. The present report deals with the purification of the enzyme and a study of its properties.

Mycobacterium tuberculosis avium (strain Takeo) was grown in Sauton medium for 5 days. The washed cells were frozen and powdered with dryice, and then poured into 20 volumes of acetone previously cooled to $-30°$. After brief stirring, the cells were allowed to settle, and were then filtered. The filter cake of the cells was desiccated in vacuo. A portion of these acetone dried cells was finely ground by 4 portions of quartz sand, and subsequently extracted with 50 volumes of 0.02 *M* sodium bicarbonate. After aging overnight in an icebox, the cells were centrifuged off at 3,000 r.p.m. for 30 minutes and the supernatant fluid was recentrifuged at 10,000 r.p.m. for 20 minutes at 0°. The supernatant fluid was then brought to 80 per cent saturation with solid ammonium sulfate. After centrifugation, the supernatant fluid was discarded. The precipitates were dissolved in 0.02 *M* sodium bicarbonate. Solid ammonium sulfate was next added to the above enzyme solution at 0°. The fraction was collected between 30 and 60 per cent saturation. The precipitates, taken up in 0.02 *M* sodium bicarbonate, were stable for several months if stored in the frozen state.

The acetyl-phosphate formed from ATP and acetate as initial reactants, was determined by the hydroxamic acid method of Lip-

mann and Tuttle (*5*) (Reaction 2):

$$\text{Acetyl-P} + NH_2OH \longrightarrow \text{Acetohydroxamate} + \text{Phosphate.} \qquad (2)$$

To a small test tube were added the following components (in micromoles): $MgCl_2$, 10; Na acetate, 100; ATP-Na, 5; hydroxylamine, 600; and water if necessary to make a final volume of 3.0 ml. after the addition of the enzyme.

The data of the hydroxylamine-trapped experiments showed that the maximum rate of the hydroxamic acid formation was obtained on simultaneous addition of ATP and Mg ions (Table I). The concentrations of each reactant were varied over a wide range, and their effect on the rate of the formation of hydroxamic acid was determined. From the results obtained, the following Michaelis constants could be determined:

$K_m = 1.3 \times 10^{-2}\ M$ for acetate,
$K_m = 3.5 \times 10^{-2}\ M$ for propionate,
$K_m = 0.9 \times 10^{-3}\ M$ for Mg ion,
$K_m = 1.7 \times 10^{-3}\ M$ for ATP.

TABLE I

Component Study of Acetokinase with Hydroxylamine as Acetyl Acceptor

Additions	Hydroxamate formed
Enzyme*	0.04 μM
Enzyme*+Acetate(100 μM)	0.04
Enzyme*+Acetate(100 μM)+ATP(5 μM)	1.10
Enzyme*+Acetate(100 μM)+ATP(5 μM)+Mg^{++}(10 μM)	2.27

* Partially purified enzyme (see text), 0.5 ml.; pH 6.8, 38°, 120 minutes.

This enzyme was strongly inhibited by some of the so-called SH-reagents, in particular by mercuric ions and *p*-chloromercuribenzoate.

In summary, the results indicate that the enzyme described above catalyzes the phosphorylation of acetate by ATP. Moreover, the enzyme obtained from *Mycobacterium tuberculosis avium* has the same properties as that found in *Escherichia coli* by Rose *et al.* As stated in our previous report (*6*), with the extracts of the same strain grown in glycerol-bouillon medium, little or no phosphorylation of acetate was observed; however

the enzyme preparation purified free from esterase was highly active in enzymatic formation of hydroxamic acid from normal valeric acid and hydroxylamine.

We are indebted to Prof. Dr. S. Akabori for his valuable advice and encouragement during the course of this study.

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